THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XI DECEMBER, 1930 No. 6

MELIOIDOSIS, WITH SPECIAL REFERENCE TO THE DISSOCIATION OF *BACILLUS WHITMORI*.

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Forwarded for publication August 1st, 1930.

WHITMORE (1913) reported a glanders-like disease occurring in Rangoon, and he gave a description of the causal organism, which has since become known as *B. whitmori*. The disease was next recognized in Malaya by Stanton and Fletcher (1921), who proposed for it the name "melioidosis." More recently the infection has been discovered in Indo-China (Pons and Advier, 1927) and Ceylon (Denny and Nicholls, 1927). It appears to be principally a disease of rats and other rodents, but it has been found in cats and dogs. Stanton, Fletcher and Symonds (1927) isolated the organism from a transient nasal discharge of a horse; but attempts at experimental infections showed that the horse is resistant to generalized infection.

MELIOIDOSIS OF A COW.

I received in 1928 a test-tube of pus from the Government Veterinary Surgeon, who had received it from another veterinary surgeon with the statement that it was from a large splenic abscess in a cow which had died suddenly from the abscess bursting into the peritoneal cavity. It was stated that the cow had been milked on the day previous to its death and then appeared in good health. *B. whitmori* was grown from this pus in pure culture. Guinea-pigs inoculated with the pus and with the culture developed typical melioidosis, including Straus's reaction of the testicles of the males. The organism was recovered in pure culture from the blood, spleens and livers of these animals.

The virulence of this strain of *B. whitmori* was tested for healthy cattle. A Sind cow with calf at heel was obtained and they were kept under observation for three weeks, when they were injected respectively with 3 c.c. and 1 c.c. of a 48-hours' broth culture. Their temperatures were taken daily and the milk of the cow was examined bacteriologically every three days. There was slight pyrexia in both animals after 24 hours, but this had subsided by

48 hours, and thereafter neither the cow nor the calf showed any general signs of ill-health.

Swellings about the size of hens' eggs developed at the sites of inoculation and slowly subsided. Some swelling and induration was present after 6 weeks. Nine months later a small contracted subcutaneous scar could be felt at the sites of inoculation by careful palpation. *B. whitmori* was never isolated from the cow's milk.

A six-months-old bull calf was inoculated with 5 c.c. of a 48-hours' broth culture of this strain. A swelling developed at the site of inoculation and became as large as a tennis ball; this was opened five weeks after the inoculation and thick caseous pus was pressed out. *B. whitmori* grew in pure cultures from this pus. The abscess continued to discharge for three weeks and then the sinus closed up, and six months later there was only a little thickening at the site of the abscess. The animal grew normally and never showed general signs of ill-health.

It may be concluded that local cattle have little susceptibility to melioidosis, but that abscess formation may occur from the infection.

THE DISSOCIATION OF *B. WHITMORI*.

Stanton and Fletcher (1924) have described two forms of growth of *B. whitmori;* one form they call "corrugated" and the other form "mucoid." Apart from the appearances of the growths on glycerine agar, they describe no other differences between these variants, and state that they "are serologically identical with one another."

When a nutrient agar culture of *B. whitmori* is plated the colonies are usually of one kind; in 24 hours each has a dense granular appearance, and after 48 hours the rim of the colony has become thickened and ridges extend out to this rim from a raised centre. Each colony is tenacious, and can be lifted entire with a platinum loop from the surface of the medium. These are colonies of the rough variant (Fig. 1). Subcultured on to glycerine agar the growth becomes very heaped up and corrugated, and after a time resembles the growth of old cultures of *B. tuberculosis*.

Occasionally other colonies appear upon the plates; they are translucent, regular, convex, smooth and not tenacious; they are colonies of the smooth variant (Fig. 3).

The rough variant is much more stable than the smooth, and can be further stabilized by incubating a broth culture for 24 hours at 37° C. and then placing it in a water-bath at 46° C. for 24 hours, and repeating this subjection to different temperatures two or three times. Subcultures on

EXPLANATION OF FIGURES.

Fig. 1.—Colony of rough variant (4 days' growth).
Fig. 2.—Colony of "intermediate" variant (4 days' growth).
Fig. 3.—Colony of smooth variant (4 days' growth).
Fig. 4.—Secondary colonies on smooth variant.
Fig. 5.—Secondary growth on smooth variant.
Fig. 6.—Broth culture of smooth variant.
Fig. 7.—Broth culture of rough variant.
Fig. 8.—Plate cultures showing rough and smooth colonies.

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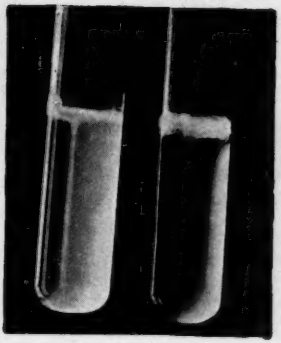

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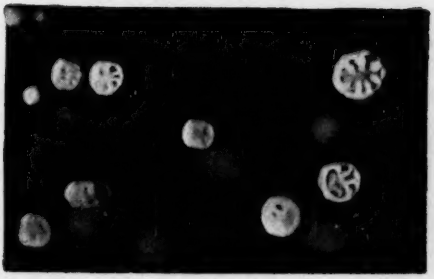

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nutrient agar then give a dull parchment-like growth in 24 hours, and this growth becomes finely and irregularly ridged in 48 hours.

The smooth variant has a marked tendency to dissociate to the rough variant, especially when grown in liquid media, so much so that I have not yet obtained a stable form which shows no dissociation after it has grown in large quantities (150–200 c.c.) of liquid media for 4 days. Plates of these cultures usually show not more than 2 to 5% of rough colonies. Cultivation on fairly dry nutrient agar slopes appears to increase the stability of the smooth variant.

There have appeared on the plates of the rough variant several other types of colonies. One of these is a parchment-like colony, with a raised rim but no ridging; another is opaque and smooth with a raised centre (Fig. 2). When these colonies are plated they usually give rise to a mixture of smooth and rough colonies; they are therefore intermediates.

Colonies which appear on plates of the smooth variant vary in size and translucency. The largest and most translucent when subcultured on to nutrient agar tends to die out, so that at the end of 10 days or less the subcultures are almost sterile, and a platinum loopful of the bacterial mass when spread upon an agar plate gives rise to a few colonies only. These colonies become yellow in a few days.

The term "suicidal" culture has been used by Hadley (1927) for other bacteria, and it will be used for these cultures of the smooth variant. When "suicidal" cultures are kept at room temperature (28° C. in the tropics) for two weeks or more, invariably secondary colonies appear, and these are denser than the primary growth and are deeply yellow. These yellow colonies often first appear on the upper part of the agar slant, and sometimes this secondary growth is a continuous streak spreading down the primary growth (Figs. 4 and 5). Subcultures from these secondary yellow colonies do not have the same tendency to die out as the "suicidal" variant. The most opaque colonies on plates of the smooth variant give rise to both rough and smooth colonies when plated, and therefore are intermediates.

Cultures of the rough variant differ from the smooth variant morphologically, culturally and biochemically; the biochemical differences may be more a matter of degree than of kind, and, as will be appreciated from the following descriptions, the experimental results have not given quantitative uniformity.

Morphology.—The rough variants appear in 24-hour-old agar cultures as short oval rods with marked bi-polar staining. They stain fairly uniformly and shadow forms are uncommon. The smooth variants are somewhat longer and narrower than the rough variants; they do not show bi-polar staining. There is marked palisading and bacilli which stain lightly, and shadow forms which are just perceptible, are common. These forms may indicate a stage of autolysis.

Broth cultures.—The rough variant forms a thick pellicle in 24 hours on broth cultures; this pellicle increases in thickness and grows up the sides of the tube. The pellicle is tenacious, and when teased with a platinum loop it can be lifted entire from the surface of the medium. The medium beneath the pellicle is not so cloudy as in similar cultures of the smooth variant (Fig. 7).

The smooth variant forms very little pellicle in 24 hours, but there is marked pellicle-formation in 48 hours, though it is never as thick as with the rough variant, and it can only be picked up with a platinum loop as small fragments. There is a dense growth beneath the pellicle (Fig. 6).

Production of alkalinity.—The rough variant in broth culture produces little or no alkali, whereas broth cultures of the smooth variant become markedly alkaline. Two conical flasks of 600 c.c. capacity and containing 150 c.c. each of lemco broth of pH 7·5 were inoculated respectively with the rough and smooth variants. They were incubated for 4 days at 37° C., when the flasks were heated to 80° C. for 10 minutes and filtered through Seitz filters. The clear filtrates had the following hydrogen ion concentrations respectively:

Rough variant	pH 7·4
Smooth variant	pH 8·4

This production of alkali appears to depend upon an ample supply of oxygen, because if the surface of the medium is covered with a layer of liquid paraffin very little alkali is produced. Thus in one experiment the results after 6 days' growth were:

Rough variant	pH 7·5
Smooth variant	pH 7·6

Production of oxalates.—The addition of a solution of calcium chloride to a filtered broth culture of the rough variant produces a heavy precipitate which is insoluble in acetic acid. Many calcium oxalate crystals are to be seen in this precipitate; sometimes they are present to such an extent that the entire precipitate appears to consist of these crystals. Similar cultures of the smooth variant give a very small precipitate and sometimes only a faint cloud.

The amount of oxalates produced in broth has been estimated by a modification of a method found satisfactory by Elsdon and Stubbs (1930) for medico-legal cases of oxalic acid poisoning. The following is the method that has been used:

A conical flask of 600 c.c. capacity and containing about 180 c.c. of lemco broth is inoculated with the variant to be tested. After the desired period of incubation, 25 c.c. of concentrated hydrochloric acid are added to the flask, which is heated to 80° C. for 10 minutes; an estimated quantity of strong ammonia solution is added so that the acidity of the contents of the flask is approximately equivalent to 1% HCl. If the neutralization is carried much further calcium oxalate crystals form, due to the calcium originally present in the culture media, and will be removed by subsequent filtration. The contents of the flask are strained through cotton-wool to remove large bacterial masses, and then filtered through a Seitz filter (as these filters are silver-plated hydrochloric acid has little action on them, but it is advisable to film the metal parts with vaseline). The clear filtrate is measured, neutralized with ammonia, and $CaCl_2$ and acetic acid added. It is allowed to stand for 24 hours; the precipitate is then removed by centrifuging in 100 c.c. tubes. The supernatant fluid becomes clear after 5 minutes' centrifuging at 3000 r.p.m. The deposit is dissolved in warm water containing 15% of concentrated hydrochloric acid. The solution is neutralized with ammonia, acidified with acetic acid and a little $CaCl_2$ solution added. The precipitate is spun out in 10 c.c. centrifuge

tubes, washed several times in distilled water, and finally washed into a weighed platinum dish, dried in an oven at 100° C. and weighed as CaC_2H_4, H_2O. (It has been found that centrifuging out the precipitate is more satisfactory than filtration through paper, because it occupies less time and the precipitate is not quickly soluble even in 15% hydrochloric acid; hence the uncertainty that all the precipitate has been dissolved from the filter-paper.)

This method is also a very delicate test for the presence of oxalates, for when filtered cultures of the smooth variant have given a scarcely perceptible cloud with calcium chloride and acetic acid the centrifuged deposit has always shown microscopically the presence of calcium oxalate crystals.

The determination of the amount of oxalates in broth cultures has always been accompanied by the determination of the amount of dissociation which has taken place; and for this purpose the broth culture is shaken and a platinum loopful is transferred to 10 c.c. of normal saline, which is well shaken. One loopful of this dilution is spread upon the surface of nutrient agar plates which have been dried in an incubator. The colonies are counted on the second day of growth.

The rough variant has not shown dissociation in these tests, whereas plates from the cultures of the smooth variant have always shown a few rough colonies; usually the number of rough colonies has been between 2 to 5%, but occasionally much greater dissociation has taken place.

The small quantity of oxalates produced in broth cultures of the smooth variant may be due to dissociation to rough and intermediate variants. Typical examples of these determinations are given in the following table:

Variant inoculated.	Days of growth.	CaC_2O_4,H_2O (gm. per 100 c.c).	Percentage of R and S colonies.
Rough	4	0·171	Rough only.
Smooth	4	0·009	95% smooth.
Rough	4	0·24	Rough only.
Smooth	4	0·012	98% smooth.
Rough	6	0·335	Rough only.
Smooth	6	0·105	58% smooth.

The results have not been very uniform, and all that can be concluded definitely is that a relatively large amount of oxalates is produced in broth inoculated with the rough variant in comparison with the small amount produced in broth inoculated with the smooth variant.

Gold chloride is reduced when boiled in broth cultures of the rough variant; this is due to the oxalates.

A free supply of air is necessary for the production of oxalates, for if the cultures are grown in broth the surface of which is covered with liquid paraffin only a small trace of oxalates is produced, although a considerable pellicle of growth takes place between the two liquids. The presence of 2% of glucose in the broth also prevents the production of oxalates.

Liquefaction of gelatine.—Gelatine is liquefied by both the rough and smooth variant, but the former is more active than the latter. When broth containing 15% of gelatine is inoculated with the rough variant and incubated at 37° C. a thick pellicle grows in 24 hours, and when the culture is set in iced water there are signs of liquefaction immediately under the pellicle. This

proteolysis proceeds rapidly, and complete liquefaction of the contents of the tube occurs in 3 days.

The action of the smooth variant is more variable, and signs of liquefaction are seldom seen before the third day of incubation and it is not complete in less than 6 days.

Free access of air is not necessary because liquefaction will take place under liquid paraffin.

Gelatine broth to which 3% of glucose has been added is not liquefied by the rough variant in 10 days, but the smooth variant produces liquefaction in this time. This appears anomalous, and possibly the more rapid production of acid from the glucose by the rough variant than by the smooth inhibits the proteolysis. Thus 10 c.c. of a rough variant glucose gelatine culture after 10 days' incubation was neutralized to litmus by 4·1 c.c. N/10 NaOH, whereas the figure for a similar culture of the smooth variant was 3·2 c.c.

Action in litmus milk.—Litmus milk inoculated with the rough variant shows acidity and clot in 24 hours and signs of advanced peptonization in 3 days. A similar culture of the smooth variant shows no change for 2 days and then the culture becomes acid and clots about the third day. Peptonization appears later, but never proceeds as fast as with the rough variant. If the litmus milk is covered with a layer of paraffin the action of both variants is much delayed.

Virulence of the variants.—*B. whitmori* retains its virulence in subcultures indefinitely. Cultures which have been subcultured weekly for over 4 years without passage through animals are as virulent or nearly as virulent as when the organism was first isolated.

If either variant is injected intravenously into a rabbit in a dose of approximately one million organisms death occurs within 2 to 4 days, and plate cultures from the blood and tissues show colonies of the rough variant only (the more chronic types of the disease are not considered here).

It might be concluded that because the rough variant only grows in cultures from animals which have died of the septicæmic form of melioidosis, the rough is the virulent variant. But frequently the number of colonies which grow are so few as to be out of all proportion to the severity of the symptoms shown by the animal during its life and to the rapidity of its death.

SUMMARY.

1. A splenic abscess in a cow due to *B. whitmori* is reported.
2. The rough and smooth growths of *B. whitmori* are described.
3. The production of oxalates by *B. whitmori* is reported.

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THE IODINE CONTENT OF THE BLOOD OF PATIENTS SUFFERING FROM CANCER.

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*From the Department of Pathology and Bacteriology, University of Leeds.**

Received for publication September 2nd, 1930.

AMONG the large number of attempts that have been made to account for the appearance of malignant growths, there has appeared the theory that iodine deficiency is a cause of cancer. This view is founded on the claim that the blood of patients suffering from cancer always contains notably less iodine than that of healthy persons. From this it is also deduced that the determination of the iodine content of the blood in cases of suspected cancer must constitute a reliable diagnostic sign.

Apart from a few figures put forward in support of this theory, no results of determinations of blood iodine in malignant disease appear to be available. An investigation has, therefore, been carried out on this subject to ascertain how far the basic claim on which the theory rests can be substantiated.

It is obvious that for the purposes of such an investigation a knowledge of the iodine content of the blood of healthy persons is necessary. Existing work on this subject is well summarized by Orr and Leitch (1929), who show that considerable variation exists between the results obtained by different workers.

In view of this variation, it is clear that for the purposes of the present investigation no previously published figure could be accepted as a normal standard. The only rational procedure was to make determinations of the iodine content of healthy individuals with the same method and under the same conditions as were to be used for similar determinations on patients suffering from cancer. Under such uniform conditions for normal and pathological cases, and where in addition all determinations are carried out by the same individual, it is reasonable to use the values obtained for comparative purposes, even though the absolute values obtained might differ somewhat from those obtained by other workers.

METHOD.

The method which has been used for the determination of iodine in blood is that of Leitch and Henderson (1926), which, besides being one of the most recent, appears to be best adapted to the needs of the present investigation.

In the large majority of determinations 10 c.c. of blood were used, but in a few cases smaller quantities had to be employed; in no case was the volume

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less than 5 c.c. The volumes used correspond with those recommended by the authors of the method (viz. 5 c.c. or 10 c.c.).

Considerable care was taken to obtain iodine-free reagents, and in sufficient quantity to meet the needs of the whole investigation; no renewal of any reagent or solution was necessary, so that the quality of these was perfectly uniform throughout. Re-standardization of standard solutions was carried out from time to time.

RESULTS ON NORMAL INDIVIDUALS.

It has been shown by a number of workers that the iodine content of the blood of the female is affected by the sexual cycle, a rise occurring during the week preceding menstruation followed by a fall after menstruation has commenced. On this account it was decided to avoid possible variations from this cause by using males only in the investigation. As will be indicated later, this intention was subsequently modified, but was adhered to so far as normal cases are concerned.

Twelve normal cases were investigated. These comprised members of the hospital staff, medical students and laboratory assistants, whose ages lay between 20 and 40 years. The results obtained varied from 3·6 to 5·1γ (γ = one-millionth of a gramme per 100 c.c.). In addition, one hospital patient (aged 53) not suffering from malignant disease gave a result of 5·2γ.

The mean of these thirteen cases is 4·3γ.

Orr and Leitch, using the same method, found a mean value of 6γ in sixteen normal individuals. Dr. Leitch has kindly supplied me with further particulars regarding the sixteen normal individuals referred to. These are as follows:

Normal males, thirteen, of whom five had 4γ, two had 5γ, two had 6γ, two had 8γ, one had 9·5γ and one had 10γ.

Normal females, three, of whom two had 5γ and one had 9γ.

It will be seen that nine out of these sixteen cases gave results of 4 or 5γ, and therefore agree closely with the results found in my own series. It will also be noticed that Dr. Leitch's and my own figures are in agreement in indicating a lower normal limit of approximately 4γ. The difference between the two series of results lies in the range indicated between maximum and minimum values. This, after all, is not very surprising, in view of the fact that both series are small, and in my own series the individuals dealt with fall within a comparatively narrow age-period.

RESULTS ON PATIENTS WITH CANCER.

Twenty-four cases of this kind have been investigated. In none was there any doubt clinically of the nature of the condition. Most of the cases were well advanced; in only two (Nos. 5 and 17) was radical cure attempted by removal of the growth. Four cases (Nos. 3, 9, 19 and 21) were considered to be too far advanced to justify any treatment. In the remainder (except No. 2) palliative operation or radium treatment was employed. As a result of the advanced nature of the cases and the methods of treatment employed, it has only been possible to obtain material for microscopic examination in five of

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Twelve normal cases were investigated. These comprised members of the hospital staff, medical students and laboratory assistants, whose ages lay between 20 and 40 years. The results obtained varied from 3·6 to 5·1γ (γ = one-millionth of a gramme per 100 c.c.). In addition, one hospital patient (aged 53) not suffering from malignant disease gave a result of 5·2γ.

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the cases (Nos. 5, 12, 17, 22 and 24). In two cases (Nos. 1 and 3) evidence of the condition—carcinoma of the stomach—was given by the results of fractional test-meal examinations, and was confirmed in the case of No. 1 by subsequent operation; in case No. 2 post-mortem evidence was obtained.

In nineteen of the cases, blood for iodine determinations was taken before any treatment whatever had been undertaken. In three cases (Nos. 4, 5 and 6) blood was taken after a palliative operation, the growth still remaining *in situ*. In case No. 8 deep X-ray therapy had been employed but had been discontinued for about a month before blood was taken, and in the remaining case (No. 2) blood was taken from the heart a few hours after death.

While it was originally intended to use males only, it was later felt to be inadvisable to exclude breast and uterine cancer from the investigation. Consequently, eight females are included in the twenty-four cases dealt with. Of these, seven had passed the menopause, while in the case of the eighth (No. 20) blood was taken midway between menstrual periods to avoid the changes occurring immediately before or after the onset of menstruation.

Details of the cases and results of iodine determinations are as follows:

Males.

1. A. C—, aged 42. *Carcinoma of stomach.*

Fractional test-meal report: "There is complete achlorhydria with a very high total acidity and marked delay in emptying. Altered blood is present throughout."

Operative findings and procedure: Hard, irregular mass in pyloric end of lesser curvature of stomach almost completely encircling pylorus, especially posteriorly; adherent to pancreas. Hard, enlarged lymphatic glands of subpyloric, gastro-colic and especially coronary groups. Posterior gastro-jejunostomy performed.

Iodine, less than 1γ.

2. T. W—, aged 61. *Carcinoma of stomach.*

Post-mortem findings: Carcinoma of stomach; secondary deposits widespread, including liver, glands and skull.

Iodine, 4·5γ.

3. J. S—, aged 58. *Abdominal carcinomatosis.*

Fractional test-meal report: "There is rapid emptying of the stomach; complete achlorhydria; altered blood present throughout."

Examination: Three masses palpable in abdomen, viz. an indefinite lump with hard surface in left subcostal region, believed to be at or near the pylorus, one in the left iliac region and one just to the right of the umbilicus. Patient much wasted.

Iodine, less than 1γ.

4. A. W. K—, aged 69. *Carcinoma of colon: hepatic flexure.*

Operative findings and procedure: Hard, fixed mass in colon just distal to hepatic flexure. Cæcum, ascending colon and terminal portion of small intestine distended and hypertrophied; glands widely involved.

Cæcum anastomosed to transverse colon.

Iodine, 4·9γ.

5. G. P—, aged 66. *Carcinoma of ascending colon.*

Operative findings and procedure: Annular, fixed growth in ascending colon; free serous effusion in peritoneal cavity; cæcostomy performed.

Later, colectomy performed.

Microscopic examination: "The growth is typical adeno-carcinoma."

Iodine, 6·4γ.

6. W. J—, aged 60. *Inoperable carcinoma of rectum.*

Symptoms: Loss of weight, constipation, blood and much mucus in stools.

Rectal examination: A mass can be felt in the anterior wall of the rectum which is attached to the bladder.

Operation: Inguinal colostomy.

Iodine, 1·1γ.

7. E. G—, aged 49. *Carcinoma of rectum.*

Examination under anæthetic: Large inoperable growth attached to bladder.

Operation: Colostomy.

Iodine, 1·0γ.

8. J. W—, aged 59. *Carcinoma of prostate with secondary growth in femur.*

History: Patient admitted on February 19th, 1929, with enlarged prostate and pressure neuralgia. Prostate found to be hard and firm, and considered to be malignant and inoperable. Deep X-ray therapy applied until February 12th, 1929. Patient readmitted on January 8th, 1930, with spontaneous fracture of femur.

X-ray report: "Femur—fracture of upper third in large area of neoplastic bone."

Iodine, 3·4γ.

9. H. R—, aged 65. *Carcinoma of œsophagus.*

Symptoms: Dysphagia for eighteen months; much wasting.

Œsophagoscopic examination: "There is an annular growth about 12 in. from mouth; not ulcerated."

Iodine, 3·0γ.

10. G. W. K—, aged 60. *Carcinoma of œsophagus.*

Symptoms: Difficulty in swallowing solid food, and later of liquid food also. Pain over stomach and sternum; wasting.

X-ray report: "There is obstruction to solid cachet at level of bifurcation of trachea; fluids pass through. *Nil* in mediastinum."

Operation: Gastrostomy.

Iodine, 6·6γ.

11. F. M—, aged 52. *Epithelioma of tongue.*

Examination: Hard, fixed, ulcerated mass at right root of tongue, reaching as far as anterior third. It is invading fauces, tonsils and lower jaw.

Treatment: Radium inserted.

Iodine, 1·3γ.

12. T. B—, aged 60. *Epithelioma of tongue.*
Examination: Hard, fixed growth on underside of anterior third of tongue (left).
Treatment: Radium inserted; portion removed for examination.
Microscopic examination: "This shows epitheliomatous growth."
Iodine, 5·7γ.

13. J. R—, aged 62. *Epithelioma of tongue.*
Examination: Large, raised growth near root of tongue on right side.
Treatment: Radium inserted.
Iodine, 4·2γ.

14. J. P—, aged 69. *Epithelioma of lip.*
Examination: Growth extenting for about 1¼ in. along centre of lower lip; hard and fixed; typical epithelioma.
Treatment: Radium inserted.
Iodine, 1·6γ.

15. R. M—, aged 58. *Epithelioma of floor of mouth.*
Examination: Uneven, hard, fixed swelling in anterior part of mouth, on under-surface of tongue, spreading along each side of lower alveolar border. Enlarged glands below and in front of angle of jaw on right side.
Treatment: Radium inserted.
Iodine, 3·3γ.

16. B. H— aged 74. *Epithelioma of right lower jaw.*
Examination: Typical hard, fixed, irregular growth, slightly ulcerated, extending from about level of molar teeth on to fauces.
Treatment: Radium inserted.
Iodine, 3·7γ.

Females.

17. M. J. B—, aged 55. *Carcinoma of left breast.*
Examination: Hard mass 3 to 4 in. in diameter in lower quadrants of left breast; irregular, partially lobulated.
Operative procedure: Rodman's operation; insertion of radium.
Microscopic examination: "The tumour is a large encephaloid carcinoma; the glands are invaded."
Iodine, 4·8γ.

18. M. A. W—, aged 55. *Carcinoma of right breast.*
Examination: Typical carcinoma; hard, fixed, with retraction of nipple.
Operative procedure: Radium inserted.
Iodine, 6·5γ.

19. H. W—, aged 62. *Carcinoma of right breast.*
Examination: Very hard mass, adherent to skin and firmly adherent to chest-wall, just below and to outer side of nipple; beginning to ulcerate.
Iodine, 3·4γ.

20. L. R—, aged 44. *Carcinoma of chest-wall following removal of breast.*
(Left breast removed in 1926.)

Examination: Large, fixed, hard lump on left side of chest about 3 in. in diameter and about 1½ in. in depth at highest point. Two or three small axillary glands can be felt.

Treatment: Radium inserted.

Iodine, 3·6γ.

21. M. L. W—, aged 60. *Carcinoma of chest-wall.*

(Three previous operations for carcinoma of breast.)

Examination: Hard, irregular nodules on left chest-wall.

Iodine, 5·7γ.

22. A. D—, aged 52. *Carcinoma of cervix uteri.*

Operative findings and procedure: The cervix is replaced by a conical ulcer as large as a hen's egg. The growth is adherent to walls of pelvis to left and behind. A large amount of black slough was removed from the cavity. The growth is absolutely fixed, and advanced.

Radium introduced and curettings taken.

Microscopic examination: "The curettings show typical carcinoma with some œdema."

Iodine, less than 1γ.

23. K. B—, aged 43. *Carcinoma of cervix uteri.*

Examination *per vaginam*: Irregular, ulcerated mass involving whole of vaginal cervix; extends into left lateral fornix anteriorly and posteriorly, but not into right.

Menopause three years ago.

Treatment: Radium inserted.

Iodine, 10·5γ.

24. L. S—, aged 47. *Carcinoma of cervix uteri.*

Examination *per vaginam*: There is a large ulcer at upper part of vagina. It involves the whole of cervix and anterior and posterior fornices. There is some proliferating growth which bleeds easily on contact with the finger. Growth fixed to left side of pelvis by a narrow infiltrating mass.

Menopause 5 years ago.

Treatment: Radium inserted and portion of growth removed for examination.

Microscopic examination: "This is a squamous carcinoma."

Iodine, 6·7γ.

Mean of results on 16 males	...	3·2γ.
Mean of results on 8 females	...	5·1γ.
Mean of all results ...	...	3·8γ.

DISCUSSION.

Compared with the range found in the normal cases already described, viz. 3·6γ to 5·2γ, the cancer results may be grouped as follows:

Below the normal range . . .	11 cases.
Within the normal range . . .	6 „
Above the normal range . . .	7 „

or, if one accepts the implication of Dr. Leitch's figures, that normal individuals may show values for blood iodine in excess of those met with in my own series, the results can be re-stated as follows:

Below the normal range . . .	11 cases.
Within the normal range . . .	13 „

Whichever grouping is taken it is clear that there is no uniform reduction in the blood iodine in cases of cancer, since a value below what may be considered normal was only found in rather less than half the cases examined. A wider variation in the results from cancer patients than from healthy individuals is certainly evident, but in view of their much wider variation in general physical condition, this can hardly be unexpected. The fact that the average result is lower than normal, together with the fact that 11 of the 24 cases taken individually show results below normal, is of considerable interest, and might be taken to indicate a tendency towards reduction of blood iodine in cancerous conditions, but in considering this indication, certain other facts must also be taken into account. In the cases showing the lowest results, viz. Cases 1, 3 and 22, marked constitutional disturbance was present in addition to the local condition. Case 1 was extremely emaciated and anæmic, and the same was true to a large extent of Cases 3 and 22. In all, malignant disease was very advanced. It does not seem unreasonable to suggest therefore that the very low results found in these cases might be due to the poor physical condition resulting from the activities of the growth. The reverse appears to hold with respect to the cases giving high results. Thus, of those giving values exceeding 6γ, viz. Nos. 5, 10, 18, 23 and 24, all except No. 10 were in good general condition. No. 10 showed some wasting, probably due to starvation resulting from the mechanical obstruction of the growth rather than to the direct effect of the growth itself.

Certain previously ascertained facts may also have some bearing on the subject. Thus Holler and Singer (1923) have shown that all inflammatory foci store iodine. Holler (1923) quotes the observation by v. d. Velden that tumour tissue was rich in iodine, but he himself found that it was only in the case of tumours which were breaking down that there was increased affinity, this being due to secondary inflammatory changes. No mention is made of the change in the blood in such conditions, but where iodine is stored in a tumour it is reasonable to suppose that this may occur at the expense of the iodine in the circulation, and may therefore be a cause of low blood iodine in such cases. Certain of the cases in the present series, notably Cases 11, 14 and 22, which show low iodine results, had reached the stage at which ulceration was considerable.

Hence, while there may be some tendency to a reduction of blood iodine in cases of cancer, particularly when the growth is advanced, it is at least possible that where low values do occur the reduction may be due to the effect of the growth on the general nutrition or metabolism of the body, or to the effect of secondary inflammatory changes in the growth which lead to the storage of iodine within it, and consequently to withdrawal of iodine from the blood.

SUMMARY AND CONCLUSIONS.

The results of the present investigation show that there is no uniform reduction of the blood iodine in cases of cancer. Out of 24 cases examined only 11 show values below what may be considered as normal. This failure to find a constant association between low blood iodine and cancer is definite evidence against the view that iodine deficiency is a cause of cancer. While there may perhaps be a tendency towards lowered blood iodine in cancer, there is at least as much justification for assuming that the reduction is the result of the cancerous condition as there is for assuming it to be the cause of such a condition. Further, since in rather less than half of the cases examined was any reduction of blood iodine indicated, the determination of blood iodine as a diagnostic test for the presence of malignant disease can have no value whatever.

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ON THE DETERMINATION OF BILIRUBIN BY THE DIAZO-REAGENTS.

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Forwarded for publication September 3rd, 1930.

A. IN SIMPLE SOLUTION.

VAN DEN BERGH (1918) made a standard solution by dissolving 5 mgm. of bilirubin (Schuchardt) in 100 c.c. of chloroform. One c.c. of this solution was evaporated on the water-bath, and the residue taken up in 10 c.c. of a solution containing 3 gm. NaCl and 0·6 gm. $NaHCO_3$ in a litre of 70% alcohol. To this were added 2·5 c.c. of the diazo-reagent made as follows:

Solution A: 1 gm. sulphanilic acid, 15 c.c. conc. hydrochloric acid, and water to one litre.

Solution B: 0·5% sodium nitrite.

The diazo-reagent is made by taking 25 c.c. of Solution A and adding 0·75 c.c. of solution B.*

As the bilirubin solution in chloroform was found by van den Bergh to be unstable, he devised the following standard:

0·1508 gm. ammonium iron alum dissolved in 50 c.c. conc. hydrochloric acid with water to 100 c.c.

10 c.c. of this solution with 25 c.c. conc. hydrochloric acid and water to 250 c.c.

The colour standard was obtained by taking 3 c.c. of this solution along with 3 c.c. of a 10% ammonium thiocyanate solution and shaking out the iron thiocyanate with 12 c.c. of ether. The ether solution was found to match the azo-bilirubin solution described above.

Another artificial standard has since been described by van den Bergh and Muller (1927), which consists of 2·0 gm. anhydrous cobalt sulphate dissolved in water to 100 c.c. This is also stated to match the azo-bilirubin solution.

Fischer and Barrenscheen (1921) have criticized the van den Bergh method of estimation in certain important respects. On repeating van den Bergh's described procedure for the preparation of the azo-bilirubin standard, they found that the residue after evaporation of the chloroform did not dissolve completely in the sodium chloride, sodium bicarbonate and alcohol mixture; that more colour to the extent of 66% was obtained by adding the diazo-reagent to a chloroform alcohol mixture than was given by the solution obtainable by the van den Bergh procedure. They also found that full colour production was not reached in the time given by van den Bergh for coupling, and that complete coupling was not attained even in 10 minutes, as evidenced by their finding that on addition of water and chloroform to the test solution, free bilirubin was still extractable. They thus conclude that, useful as the method may be for clinical purposes, it yet gives no measure of the absolute amount of bilirubin in simple solution or in plasma.

Van den Bergh and Muller (1927) have subsequently advised that the chloroform standard be evaporated, not to dryness, but so that the bilirubin remains dissolved in 0·1 to 0·2 c.c. of chloroform. Apart from this concession the objections of Fischer and Barrenscheen have so far remained unanswered, and as they are of fundamental importance in the determination of bilirubin by the diazo-reagents, they obviously call for a re-examination of the subject. This I have attempted as follows:

Ten mgm. of bilirubin† were transferred to a dry 100 c.c. glass-stoppered flask and about 90 c.c. of pure chloroform added. After about a day, with occasional shaking, the substance completely dissolved. Chloroform was then

* For complete diazotization 25 c.c. of Solution A require 2·0 c.c. of Solution B. The diazo-reagent here described is substantially the same as that used by Ehrlich (1883, 1886). About two-thirds of the sulphanilic acid used remains as such, and under certain conditions some coupling occurs with the diazotized substance, giving a brownish-red colour. Such a partially diazotized reagent ensures the absence of nitrous acid, in the presence of which bilirubin is quickly changed in such a manner that it no longer couples. This action of nitrous acid may be explained on the assumption of the formation of the oxime of a pyrrole group (Fischer and Rose, 1912), or by an oxidizing action similar to that displayed by nitrous acid in the Gmelin test.

† The bilirubin used here was prepared by the Eastman Kodak Company, Rochester, N.Y., U.S.A., who kindly provided me with a description of its method of preparation. The substance was a microcrystalline bright orange powder, completely soluble in chloroform, and leaving no ash on ignition. There is no doubt that the material was of a high degree of purity.

added to the 100 c.c. mark and the contents of the flask were thoroughly mixed. Fischer and Barrenscheen found such a solution to be stable for over a year, and I can attest that it is stable for at least several months.

0·5 c.c. of this solution was transferred to a small glass basin and evaporated to dryness in a water-bath. The addition of 10 c.c. of the van den Bergh bicarbonate-alcohol mixture dissolved only a trace of the residue, and subsequent addition of 2·5 c.c. of the diazo-reagent gave only a perceptible pink colour. This amply confirms the statement of Fischer and Barrenscheen that bilirubin is practically insoluble in alcohol, and that the addition of sodium chloride and sodium bicarbonate in the concentrations mentioned does not appreciably affect the question.

To 0·5 c.c. of the bilirubin solution I next added 9·5 c.c. of 96% alcohol followed by 2·5 c.c. of diazo-reagent. A pink colour soon appeared, which changed to a deeper reddish-purple after 5 to 10 minutes. On comparing this in a test-tube side by side with the artificial standard of ethereal iron thiocyanate it was found that the colours were of almost the same intensity, as was also the standard made with cobalt sulphate, although all the solutions were of different hue. The finding of Fischer and Barrenscheen is thus confirmed, yet at the same time van den Bergh must have treated his standard chloroform solution in such a way that it was all obtained in solution.

Van den Bergh regards the method as providing an estimation rather than a quantitative determination of bilirubin in serum, and if, as stated by Fischer and Barrenscheen, the coupling is not complete, we can hardly hope for a satisfactory means of measuring the amount of bilirubin by the diazo-method. Nevertheless, it was decided to test accurately the relation of the amount of colour developed to the amount of bilirubin in the test solution. In this connection, however, certain preliminary observations should be recorded:

When coupling takes place under the conditions described above, or in presence of the negligible amount of sodium bicarbonate recommended by van den Bergh, the colour developed is appreciably more blue than the iron thiocyanate solution, and very markedly more blue than the cobalt sulphate solution. Matching colours under these circumstances is open to a relatively large error.

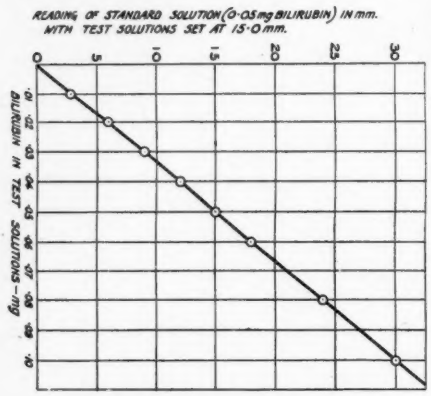
The speed of coupling is markedly influenced by the reaction of the medium. Under the above-described conditions increase of colour over a period of 5–10 minutes is quite appreciable without recourse to a colorimeter. With more acid present the reaction is even more retarded and is probably not complete. The solution may, however, be buffered to a weakly acid reaction, under which conditions the coupling is apparently practically immediate, and, as will be shown, complete. The azo-bilirubin colours are less stable in weakly acid solution than in more strongly acid solution.

The presence of relatively large proportions of chloroform in the coupling medium also retards coupling. Thus if to 0·5 c.c. of the chloroform solution of bilirubin, 2 c.c. of chloroform and 7·5 c.c. of 96% alcohol are added, followed by 2·5 c.c. of Ehrlich's diazo-reagent (under which conditions we have still a homogeneous solution), then less colour develops, and on the addition of water at the end of 5 minutes uncoupled bilirubin is found to be present in the chloroform layer.

These findings appear to account for the statement of Fischer and Barrenscheen that the coupling is not complete.

After various attempts to modify the conditions of coupling so that the reaction was likely to be rapid and complete, the following method was adopted:

A solution of 6 gm. of $Na_2HPO_4.12H_2O$ in 100 c.c. is, on the basis of one equivalent of sodium, about one-sixth normal alkali. The diazo-reagent is about one-sixth normal acid due to hydrochloric acid. On mixing an equal volume of the diazo-reagent with the disodium hydrogen phosphate solution, we obtain a weakly acid solution buffered by NaH_2PO_4. Under these conditions a faint reddish colour becomes perceptible after about 10 minutes in the diazo-solution, due probably to coupling with sulphanilic acid in the solution. For this reason the diazo-solution is made immediately before using for each pair of tests in the following procedure. For example, to 4 c.c. of the Ehrlich diazo-reagent, 4 c.c. of the disodium hydrogen phosphate solution are added, from which 2·5 c.c. were used in each test.*



The method was tested over the range of 0·1 to 0·01 mgm. of bilirubin—that is, using 1·0 to 0·1 c.c. of the standard bilirubin solution in chloroform. 96% alcohol was added to make each volume 10 c.c., and to this 2·5 c.c. of the phosphate buffered diazo-reagent were added. The colour produced from each was read in a Duboscq colorimeter against a standard containing 0·05 mgm. of bilirubin. To eliminate any error that might arise from fading, the standard was re-made with each solution to be measured. To obtain a direct proportionality between the amount of bilirubin present and the amount of colour produced, each solution, which we shall term the test solution, is placed in the left-hand cup of the colorimeter with the plunger set at a constant depth of 15 mm. The standard solution is placed in the right-hand cup and the depth of colour altered to match.

The solutions were in each case mixed and the colours produced in test-tubes. Suitable amounts of the fluids were then transferred to dry colorimeter cups and readings made without delay. The results are given in the above graph, from which it is seen that there is a strict linear proportionality between the amount of bilirubin present and the amount of colour produced from it by the diazo-reagent.

The addition of water to the azo-bilirubin solutions till the separation of the chloroform failed to reveal the presence in the chloroform layer of the

* The concentration of diazobenzene-p-sulphonic acid is here, of course, only half that used in the van den Bergh procedure, yet there is still present more than a thousand times the theoretical requirement for 0·1 mgm. bilirubin.

slightest trace of bilirubin. The evidence is thus altogether in favour of the conclusion that, under the above described conditions, bilirubin couples quantitatively with diazobenzene-p-sulphonic acid. The reagents in themselves have no colour and, as the colour produced is strictly proportional to the amount of bilirubin present, this diazo-reaction obviously supplies a method of the highest accuracy for the determination of bilirubin in simple solution. The colour obtained under these conditions from pure bilirubin matches the artificial cobalt sulphate solution much more closely than that obtained by the method of van den Bergh. The colour hues are, however, not sufficiently near to each other to admit of precise standardization, but from a number of readings it was found that when the azo-bilirubin solution containing 0·05 mgm. of bilirubin in 12·5 c.c. was set at a depth of 15 mm., the cobalt sulphate solution read about 14·2 mm., indicating that 1·9 gm. of anhydrous cobalt sulphate in 100 c.c. provides a more accurate standard than 2·0 gm. in 100 c.c. The amount given by McNee and Keefer (1925), that is, 2·161 gm. in 100 c.c., is definitely too high.*

The colour of the azo-bilirubin solution produced by the buffered diazo-reagent also closely resembles in hue that obtained from serum. Indeed the conditions of coupling here may be regarded as very similar to those obtaining in an extract from serum. Such a standard solution may be used for the determination of bilirubin in serum, but there is much to be said for the use of a stable artificial standard such as is provided by the cobalt sulphate solution.

The colour values obtained by using the chloroform solution containing 10 mg. bilirubin in 100 c.c. were checked by using another chloroform solution containing 25 mg. per 100 c.c.

B. IN SERUM.

The procedure of van den Bergh and Muller (1927) is as follow: To a centrifuge-tube add 0·5 c.c. of serum and 1·0 c.c. of 96% alcohol. Centrifuge, and remove 1·0 c.c. of the supernatant fluid to a test-tube. To this add 0·5 c.c. of 96% alcohol and 0·25 c.c. of diazo-reagent. The colour is matched with that prepared from a 1 : 200,000 solution of bilirubin, or with the artificial standards mentioned above. The amount of bilirubin present in the serum is calculated by van den Bergh as follows: On the addition of 1 c.c. of 96% alcohol to 0·5 c.c. of serum the resultant volume is found to be $\frac{10}{7}$, and the dilution thus $\frac{20}{7}$, the volume occupied by protein precipitate being neglected. By the addition of 0·5 c.c. of alcohol and 0·25 c.c. of diazo-reagent to 1 c.c. of the supernatant fluid there is a further dilution of $\frac{7}{4}$, so that the total dilution of the serum is $\frac{20}{7} \times \frac{7}{4} = 5$. That is, the value found by comparison with the standard solution must be multiplied by 5. If, for example, in the colorimeter a given depth of test

* Some care is necessary in drying $CoSO_4.7H_2O$, the usual commercial salt. When heated for 6 hours in an electric oven at about 150°, it was found that the resultant apparently dry powder still contained a relatively large amount of water. When heated in a muffle furnace beyond a very dull-red heat there is a slow decomposition of the sulphate and the production of a black insoluble oxide. The dry powder dissolves slowly in cold and easily in warm water. The standard solution is finally filtered from a trace of insoluble material.

solution matches the same depth of standard solution, there will be present in the serum a concentration of $5 \times \frac{1}{200,000}$ of bilirubin. If the test solution had only one-fifth the colour of the standard, then the concentration would be $5 \times 0{\cdot}2 \times \frac{1}{200,000}$. For convenience, van den Bergh used for such an expression as this simply the product of $5 \times 0{\cdot}2$, it being understood that it is further multiplied by $\frac{1}{200,000}$.

The method of expression is, despite this simplification, obviously a clumsy one, as a bilirubin value of, let us suppose, 10 is meaningless in itself, and in its more complete form of 1 in 20,000 it still conveys to us little, as it is an unusual form of expression of the concentration of a blood constituent. The general adoption of McNee's translation of the term "Bilirubinwert" by "bilirubin unit" is even more unfortunate, as the term "unit" appears to have been adopted in physiology, especially since the standardization of insulin, for purposes of biological assay. It thus connotes the lack of quantitative accuracy. In the writer's opinion, the simplest way is to express the concentration of bilirubin in the serum in the manner in which it has become the custom to express the concentration of other non-protein blood constituents, that is, as mgm. per 100 c.c. Had the expression originally been in this form it is unlikely that a gross inaccuracy (of 20%) in the calculations of van den Bergh could have hitherto escaped the notice of the various workers who have used his method. The error lies in the fact that van den Bergh describes his standard as one of 1 in 200,000, whereas it is actually one of 1 in 250,000 of bilirubin, the standard solution containing 0·05 mgm. of bilirubin in the form of azo-bilirubin, in a volume of 12·5 c.c., *not* 10 c.c. This means, *cæteris paribus*, that all the values in the literature to date are 20% too high.

The calculation is simply performed as follows: Since 12·5 c.c. of standard solution contain 0·05 mgm. of bilirubin, then 100 c.c. of standard contain 0·4 mgm. of bilirubin. If the total dilution of the blood-serum is taken as 1 in 5, and a given depth of azo-colour from the serum matches the same depth of colour from the standard, then 100 c.c. of serum will contain $5 \times 0{\cdot}4$ mgm. or 2·0 mgm. of bilirubin. For a normal human blood-serum at this dilution the colour would amount to about $\frac{1}{10}$ of that of the standard; that is, one would have $5 \times \frac{1}{10} \times 0{\cdot}4$ mgm. $= 0{\cdot}2$ mgm. of bilirubin per 100 c.c. In general terms the *total dilution of the blood-serum* $\times$ *ratio of the readings* $\times$ *0·4* $=$ *mgm. of bilirubin per 100 c.c. of fluid* under investigation.

Various modifications of the van den Bergh method for the determination of bilirubin in serum have been suggested. That which has received most attention is the modification of Thannhauser and Andersen (1921). This was introduced to overcome the adsorption of bilirubin frequently encountered in sera from patients with obstructive jaundice. In this method 0·5 c.c. of diazo-reagent is added to 1 c.c. of serum, followed by 2·5 c.c. of 96% alcohol and 1·0 c.c. of saturated ammonium sulphate. The object of the method is to allow

the formation of azo-bilirubin before protein precipitation, and thus eliminate adsorption. The main purpose of the incorporation of ammonium sulphate appears to be to complete protein precipitation.

From 1 c.c. of serum the total volume is thus 5·0 c.c., so that the dilution appears to be 1 in 5. It is, however, taken as 1 in 4 for the reason that only 0·2–0·3 c.c. of the ammonium sulphate goes into solution, and the volume occupied by protein precipitate may be taken as compensating approximately for that amount in excess of a 1 in 4 dilution.

Thannhauser and Andersen also used a stronger diazo-reagent, and converted the reddish azo-colour into a blue by the addition of conc. hydrochloric acid before reading. But these changes are of doubtful value and have not been generally adopted.

The main arguments for the adoption of this modification are that it simplifies the technique, and enables the worker to test at the same time whether the serum gives a "direct" reaction. In an acid medium the serum proteins do not affect the activity of diazotized sulphanilic acid, and they provide a greater buffering effect on the coupling medium, thus leading to greater similarity in colour hue than is obtained under the van den Bergh conditions. It may also be accepted that the Thannhauser and Andersen method reduces adsorption, although in the writer's experience it does not eliminate it. When the icteric serum contains a relatively large amount of bilirubin, amounts smaller than 1 c.c. of serum must be taken for the test if quantitative accuracy is desired. Apart from the question of adsorption, it is perhaps unsafe to have in serum test solutions a concentration of bilirubin greater than the maximum of the range in which I have shown that the reaction is strictly quantitative, that is, 0·1 mgm. of bilirubin in a volume of 12·5 c.c. Using the Thannhauser and Andersen method, in which 1 c.c. of serum is diluted to approximately 4·0 c.c., this means that not more than about 0·03 mgm. of bilirubin should be present in the test portion of serum. In other words, if the serum has more than 3·0 mgm. of bilirubin per 100 c.c., an amount less than 1 c.c. should be used to bring the concentration within the approved range.

In his monograph, van den Bergh had already made it clear that in bilirubin-rich sera dilution with water before the addition of alcohol leads to higher bilirubin values. This he attributed to decreased loss from adsorption, but the limit of concentration at which bilirubin actually in solution will react quantitatively with the diazo-reagent is probably also exceeded in such sera. The question of whether the van den Bergh procedure, with proper attention to dilution, gives a lower value than the Thannhauser and Andersen method under the same conditions does not appear to have been investigated. But certainly, accurate results cannot be obtained by either method without due regard to dilution.

The incorporation of ammonium sulphate appears to the writer to be of doubtful value. The additional protein precipitated by it is negligible and of no apparent advantage to the determination. The presence of indoxyl in sera from uræmic patients is not detectable by the diazo-method in the presence of ammonium sulphate. The procedure for routine work might well be simplified by adding in place of ammonium sulphate 0·3 c.c. additional alcohol, thus: 1 c.c.

of serum + 0·5 c.c. of diazo-reagent + 2·8 c.c. of 96% alcohol, giving a dilution of approximately 1 in 4.

The quantitative recovery of bilirubin added to serum is shown by the following experiment: To two centrifuge-tubes add 0·1 c.c. of chloroform containing 0·01 mgm. of bilirubin. To one tube add 1·0 c.c. of a serum practically free from bilirubin, along with 0·5 c.c. of diazo-reagent and 2·8 c.c. of 96% alcohol. Centrifuge. To the other add 3·8 c.c. of 96% alcohol and 0·5 c.c. of buffered diazo-reagent. The amount of colour developed in each case was found to be the same.

The precision with which bilirubin may be estimated in serum and similar body-fluids rests, then, with the degree of accuracy desired by the worker rather than with the principles of the method. The routine method as outlined above is relatively accurate despite the very small amounts of substance measured. A low normal bilirubin value for human blood of, say, 0·1 mgm. per 100 c.c. is, of course, open to a relatively high percentage error—perhaps 50%; but when we consider that such a value may be obtained from 0·5 c.c. of blood-serum, or with 0·0005 mgm. of the substance to be measured, we can hardly expect much better. When the amount present is greater than 0·5 mgm. per 100 c.c. the degree of accuracy then depends mainly on two factors—the measurement of the dilution, and the presence of pigments other than bilirubin in the serum. The main factors affecting the accuracy of the dilution figure are variability in the amount of protein in the serum, and change of volume on mixing alcohol and water. Interfering pigments could be compensated for by the use of such a colorimeter as that described by Bürker (1927), but for almost all practical purposes the error from pigment interference is negligible.

CONCLUSION.

The basic principles of the method for the estimation of bilirubin by the diazo-reagent have been re-examined, and details given for the precise determination of bilirubin in simple solution or in blood-serum.

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ON THE TWO TYPES OF BILIRUBIN DIAZO-REACTION IN SERUM, WITH A HYPOTHESIS ON THE NATURE OF THE BILIRUBIN IN THE SERUM FROM HÆMOLYTIC JAUNDICE.

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Forwarded for publication September 3rd, 1930.

VAN DEN BERGH and Muller (1916), when testing bile with the Ehrlich diazo-reagent, inadvertently omitted to add alcohol, and found to their surprise that the test thus succeeded just as well as in its presence. Yet a neutral sodium bilirubinate solution would not couple in the absence of alcohol. On testing horse-serum and the serum from patients with pernicious anæmia and certain types of hæmolytic jaundice, it was found that a colour was not given by the diazo-reagent alone; whereas under the same conditions, the serum from patients with obstructive jaundice gave an immediate colour just as had been found for bile. The test as obtained with bile or with serum from obstructive jaundice was thus termed a *direct*, and that given only in the presence of alcohol, an *indirect diazo-reaction.* All sera, of course, which contain bilirubin give an indirect reaction, and determinations of bilirubin are performed on protein-free alcoholic extracts. Van den Bergh and Muller suggested that hæmolytic jaundice could thus be differentiated clinically from obstructive jaundice, and postulated the existence of two types of bilirubin. At this point three main differences were evident: (1) Difference in coupling with diazo-compounds. (2) Difference in adsorption by protein precipitates: when "obstructive sera" were precipitated by alcohol, much of the bilirubin was precipitated with it, whereas this was not so for "hæmolytic sera." (3) Different speed of oxidation: serum from obstructive jaundice rapidly lost its bilirubin on exposure to air, but that from hæmolytic jaundice was very stable. We shall return to these later, as they are important in any attempt to explain the direct and indirect Van den Bergh reactions. Van den Bergh and Muller further showed that coupling is promoted by the presence of such bile-acids as glycocholic, dehydrocholic and cholic acids, but did not regard the presence of glycocholic acid in bile as providing a satisfactory explanation of its behaviour. After bile is heated for a short time it ceases to give a direct reaction, but the indirect remains. No satisfactory explanation for the difference of behaviour was, however, obtained. They suggest that in hæmolytic sera the bilirubin is bound in such a manner to protein that the combination must be broken by the addition of alcohol before coupling takes

place. In his monograph, 'Der Gallenfarbstoff im Blute' (1918), Van den Bergh reviews his work up to this date.

The chief biochemical interest in the extensive clinical literature which has grown up since that date lies in the problem of the existence of two types of bilirubin. The evidence is too strong to admit of any doubt that there are two such types. Two sera containing the same amount of bilirubin may react differently. Body-fluids such as bile, and serum from obstructive jaundice, give the so-called direct test of Van den Bergh; whereas body-fluids such as exudates, transudates and hæmorrhagic fluids do not give the direct test, but couple after treatment with alcohol. Also, presumably without a knowledge of the Van den Bergh distinction, Hoover and Blankenhorn (1916) showed that "obstructive bilirubin" easily dialyses, whereas "hæmolytic bilirubin" does not.

We may thus sum up the evidence upon which the existence of two types of bilirubin depends. We may call these the direct and delayed types, using "delayed" in reference to all reactions which are regarded as non-direct, rather than in the more restricted clinical sense. (The use of indirect in contra-distinction to direct is confusing in that both types of bilirubin, as they exist in the body-fluids, couple in presence of alcohol—that is, give an indirect test.)

Direct Bilirubin, as it Occurs in Bile or in the Serum of Patients with Obstructive Jaundice.

(*a*) Rapidly couples with diazo-compounds in an acid medium.

(*b*) Is changed to the delayed type after heating for a short time (Van den Bergh, 1918; Lepehne, 1920), or allowing to stand in an oxygen-free atmosphere (Newman, 1928).

(*c*) When exposed to an atmosphere containing oxygen (Van den Bergh, 1918), or by the use of very mild oxidizing agents (Andrewes, 1924), suffers a relatively rapid oxidation, or change of the pigment in such a way that it no longer couples under any conditions with diazo-compounds.

(*d*) Dialyses through a collodion membrane (Hoover and Blankenhorn, 1916; Blankenhorn, 1917; Brulé, Garban and Weissmann, 1922); corresponding to the general finding of bilirubinuria in obstructive jaundice, with a kidney threshold in man of 3–4 units of bilirubin (Van den Bergh, 1918).

(*e*) Is not extractable with chloroform (Grunenberg, 1922; Andrewes, 1924; Roberts, 1928).

(*f*) Is readily adsorbed by the protein precipitated from the fluid by alcohol (Van den Bergh, 1918; Thannhauser and Andersen, 1921).

(*g*) Is not readily prepared in cystalline form.

Delayed Bilirubin, as it Occurs in Hæmorrhagic Exudates and in the Serum of Patients with Hæmolytic Jaundice.

(*a*) Couples slowly or not at all with diazo-compounds. After treatment of the fluid with alcohol it reacts as direct bilirubin.

(*b*) May be converted to the direct type on addition of alkali in the proper

amount (Collinson and Fowweather, 1926; Newman, 1928), although this is questioned (Roberts, 1928).

(*c*) Is less readily oxidized (Van den Bergh, 1918; Andrewes, 1924).

(*d*) Does not dialyse through a collodion membrane (refs. as under (*d*) above); corresponding to the general absence of bilirubinuria in hæmolytic jaundice, and a much higher kidney threshold of excretion.

(*e*) Is extractable with chloroform (refs. as under (*e*) above).

(*f*) Is not adsorbed by the protein precipitated from the fluid by alcohol (refs. as under (*f*) above).

(*g*) Is readily prepared. Van den Bergh (1918) has described a simple method by which bilirubin may be prepared in crystalline form from as little as 10 c.c. of blood-serum. Fischer and Reindel (1923) confirmed these findings.

There have been various attempts to account for these two types of bilirubin. Van den Bergh (1918) suggested that the delayed type is present in the serum in combination with protein, and that preliminary treatment with alcohol is necessary to effect disruption of the union before it will couple with diazo-compounds. The experimental evidence available on the subject is against this view (Andrewes, 1924). Feigl and Querner (1919) suggested combination with a lipoid, and Rosenthal and Holzer (1921), from their general findings of hypercholesterolæmia in obstructive jaundice and hypocholesterolæmia in hæmolytic jaundice, attributed the direct reaction to the accelerating action of cholesterol. Van den Bergh and Muller (1916) noted the accelerating action of bile-salts on the coupling, as later did Thannhauser and Andersen (1921); but *in vitro* experiments of the former workers indicated that the presence of bile-salts did not account for the direct reaction, and Andrewes (1924) has advanced some evidence against the lipoid hypothesis. E. Adler and Strauss (1922) attribute the direct and delayed reactions to the hydrated or dehydrated state of the proteins in association with the bilirubin in the serum, and instance the enhancing effect on the colour of such substances as ammonium sulphate and caffein-sodium salicylate. The hypothesis of Collinson and Fowweather (1926), that direct bilirubin is present in the serum probably as an ammonium salt, whereas delayed bilirubin is present as the free acid in a colloidal condition, is certainly one of the most suggestive that has been advanced, especially if kept to the more general terms of an alkali salt. Bilirubin is a dibasic acid and may theoretically occur as such, or as the mono- or di-alkali salts. At the hydrogen-ion concentration of blood it is most probably there as a mono-alkali salt, and the assumption that the direct type is a mono-alkali salt—for example, sodium hydrogen bilirubinate—fits practically all of the findings. The assumption of the presence of colloidal bilirubin to account for the delayed type fits equally well its solubility in chloroform, inability to dialyze, and greater stability towards oxidizing agents. We shall examine these hypotheses in more detail.

In certain endeavours to explain the direct and indirect tests, it has been stated in the literature that " pure bilirubin " gives a direct test in alcohol and a delayed test in water. Bilirubin is, as a matter of fact, quite insoluble in water, and an aqueous suspension shows no sign of coupling even after standing for twelve hours in presence of the diazo-compound. An alcoholic suspension

of bilirubin, with the reagent added, shows merely a trace of azo-bilirubin after standing twelve hours. I have likewise been unable to obtain the semblance of a direct test by adding varying amounts of sodium hydroxide, still maintaining the coupling medium acid. If, however, a very small quantity of bilirubin is just dissolved in sodium hydroxide, and a few drops of 6% aqueous disodium hydrogen phosphate added, then mixing with the diazo-reagent is followed by immediate coupling in a definitely acid medium. Under these conditions it is possible to duplicate with pure bilirubin the direct reaction obtainable in serum. Similar buffering systems are present in serum, and in all likelihood the direct Van den Bergh reaction is attributable to the presence in the serum of sodium hydrogen bilirubinate. This is in accordance with the hypothesis of Collinson and Fowweather.

It is less easy to devise conditions to obtain a delayed reaction with pure bilirubin. Consideration of this problem has led the writer to believe that the behaviour of delayed bilirubin cannot be fully explained on the hypothesis that it is present in the serum simply as colloidal bilirubin. Colloidal bilirubin is presumably quite insoluble in alcohol, and the addition of alcohol to serum would not bring it into solution. In short, we must suppose that the delayed type of bilirubin is in solution in the serum, and even more securely in solution than the direct type, as there is some absorption of the latter on the addition of alcohol. We must regard the addition of alcohol to serum, not as a means for obtaining bilirubin in solution, but as a means for the precipitation of protein without precipitating the bilirubin. We may regard a chloroform solution of bilirubin as analogous to a "delayed serum." Coupling is almost negligible when the diazo-reagent is added to a chloroform solution of bilirubin (a delayed reaction), but after the addition of alcohol we obtain an "indirect reaction." This would appear a somewhat remote analogy, yet, when we find that, for example, 0·1 c.c. of a chloroform solution of bilirubin to which has been added 9·9 c.c. of alcohol, then 0·5 c.c. of diazo-reagent, reacts quantitatively (*vide* foregoing paper), whereas the same amount of bilirubin suspended in 10 c.c. of alcohol gives no colour with the reagent, the conditions then approach what we might expect to find physiologically. The bilirubin solvent —it may be a lipoid or sterol—need be present in relatively small amount to retain the bilirubin in solution on the addition of alcohol. The solvent containing the bilirubin is probably insoluble in acid aqueous solution, but soluble in alcohol. The action of alcohol in hastening coupling in the delayed serum I should thus regard, not as one of breaking any complex of bilirubin with protein or other material, but as attributable to the mutual solvent action of alcohol for water and bilirubin solvent, and should regard a chloroform solution of bilirubin as providing a crude model of a delayed serum.

On this hypothesis the bilirubin of the delayed serum is regarded as free bilirubin, or at any rate not combined with base, as is most probably the case with direct bilirubin. It may, of course, be in combination with its solvent or some other serum constituent so that it resists salt-formation and oxidation. It would not be dialysable, but extractable with chloroform. Such a hypothesis to account for the delayed type of bilirubin would appear to the writer to fit the findings better than the assumption of the presence of colloidal bilirubin.

CONCLUSION.

An attempt has been made to determine the probable nature of the two kinds of diazo-reaction for bilirubin obtainable in icteric sera. The so-called direct reaction is most probably due to the presence of sodium hydrogen bilirubinate, and the so-called delayed reaction to the presence of free bilirubin in an undetermined solvent which is soluble in alcohol.

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THE INFLUENCE OF THE INTRAVENOUS INJECTION OF UREA ON THE EXCHANGE OF SUBSTANCES BETWEEN THE BLOOD AND THE TISSUES.

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Received for publication September 15th, 1930.

THE present study is concerned with the interchanges between the blood and tissues in man—in health and in various diseases—following the intravenous injection of urea. The main line of investigation consisted of observing the effects of the intravenous injection of a known amount of urea on the urea, salt and water content of the blood and urine, at intervals for a period of two hours after the injection. Two experiments on similar lines were also made with calcium chloride in order to compare the behaviour of calcium, in view of its reputed action on the permeability of cell membranes, with that of urea. At one stage of the investigation it became desirable to know the effect

of the injection of urea on the process of urea formation within the body, and with this end in view the ammonia excretion after the administration of urea intravenously and by mouth was estimated in a number of cases.

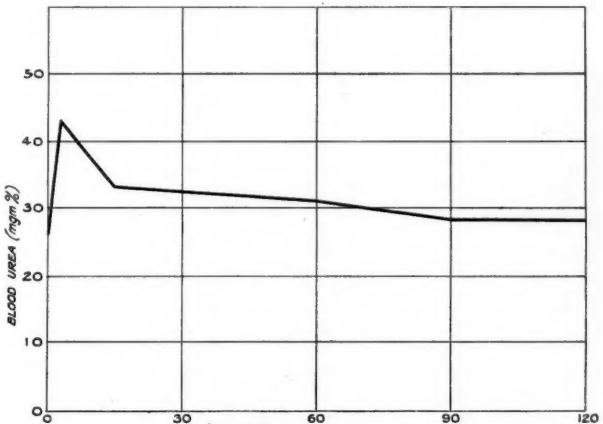
It is hoped that the results obtained will help in throwing fresh light on the rapidity of interchanges between the blood and the tissues, on the mechanism of adjustment to changes in the composition of the blood, and on the distribution of substances (and more especially of urea, salt and water) between the blood and tissues. The influence of the sudden introduction of urea into the body on urea metabolism and the bearing of this on the pathogenesis of uræmia will be discussed. It is thought that the differences in the movements of salt and water in œdematous and non-œdematous subjects following the injection afford new data for the discussion of the regulation of the volume of the blood and of the pathogenesis of œdema.

EXPERIMENTAL.

The bladder was emptied at 10 a.m., 11 a.m., 12 and 1 p.m. The first specimen was discarded, the others were kept separate. At 11 a.m. about 20 c.c. of blood was taken from a vein. 5 c.c. was placed in a citrated, the remainder in an oxalated tube. About 5 gm. of urea (the exact amount was estimated) in 10 or 20 c.c. of water was then injected intravenously, and specimens of blood were taken 3 minutes, 15 minutes, 1 hour, 1½ hours and 2 hours after the injection, each being treated in the same way as the first. The citrated blood was used for the estimation of the whole blood chlorides, as NaCl. Some of the oxalated blood was used for the estimation of the blood-urea by Maclean's method. In 17 cases the plasma urea was also estimated after time had been given for the urea to diffuse into the blood-cells, this duplicate estimation serving as a check on the results. With the rest of the blood an attempt was made to follow changes in the blood volume by measuring the volume of plasma relative to that of the corpuscles. The method we adopted was to centrifugalize about 10 c.c. of blood in a graduated tube for 15 minutes, and read off the proportions of cells and plasma. The results were unsatisfactory. Sometimes hæmolysis occurred, making them worthless. During the centrifugalization slight evaporation was apt to occur. It was thought possible that varying proportions of oxalate might affect the results by causing varying degrees of osmotic shrinking, and Capps (1903) and Hooper *et al.* (1920) state that this is so, though the latter found no strict parallelism between the concentration of oxalate and the degree of shrinking, and we detected no such result in experiments made to confirm it. Possibly different degrees of oxygenation of the blood or the osmotic changes set up by the injection might cause variations in the size of the cells, but we believe that such changes are small enough to be neglected. There are thus so many fallacies in the rationale and technique of the test that no reliance can be placed on individual blood volume curves, but the similarity in the shape of the curves is so marked that it seems reasonable to presume that they express some real truth.

The quantity of urine and its urea and salt content was estimated for each hour, and in some the amount of ammonia excreted.

Of the experiments done 29 were chosen as being reasonably free from experimental error. These were done on 6 normal persons, 3 cases of mild acute nephritis, 2 of infective endocarditis with hæmaturia, 5 of chronic nephritis, 4 of high blood-pressure, 4 on cases of various surgical diseases of the urinary tract, 2 of heart failure with œdema, and 3 of hepatic cirrhosis with ascites. Table I shows the experimental results in 6 cases selected at random, and Figs. 1, 2 and 3 show typical blood urea, blood chloride, and red-cell volume curves respectively. The blood-urea curve is essentially similar in health and in the various diseases investigated. Three minutes after the injection the blood urea has risen from 6 to 48 mgm. % above the resting level. It then falls, first quickly, then more slowly, until in most cases the resting level has nearly been reached in 2 hours. In no case



CURVE I.

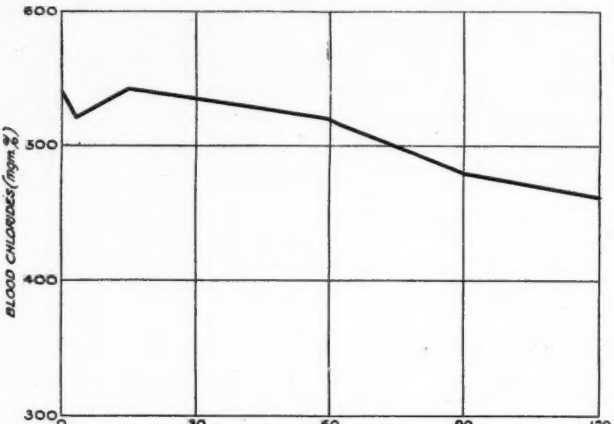
has all the urea injected been excreted in 2 hours. In the normal cases the average urea excretion in 2 hours was 2·68 gm. The average excretion in 2 hours by 16 normal persons to whom no urea was given was 2·45 gm. In the cases of acute nephritis the average amount excreted was 2·55 gm., in the cases of cirrhosis of the liver 2·1 gm., and in the chronic nephritics 1·37 gm. The greatest amount excreted in any single case was 4·61 gm. in a case of recovering acute nephritis, and the least 0·93 gm. in a case of chronic nephritis.

Three minutes after the injection of urea there is almost always a drop of 8 to 50 mgm. % in the blood chlorides. There is sometimes a further fall for 15 minutes followed by a gradual rise almost to the resting level in 2 hours, but usually there is a rise at 15 minutes almost to the resting level, followed by a slight fall for the rest of the 2 hours. The average depression below the resting level varied from 2 mgm. % in the chronic nephritics to 40 mgm. % in the cases of hepatic cirrhosis. In the normal cases it was 10 mgm. %.

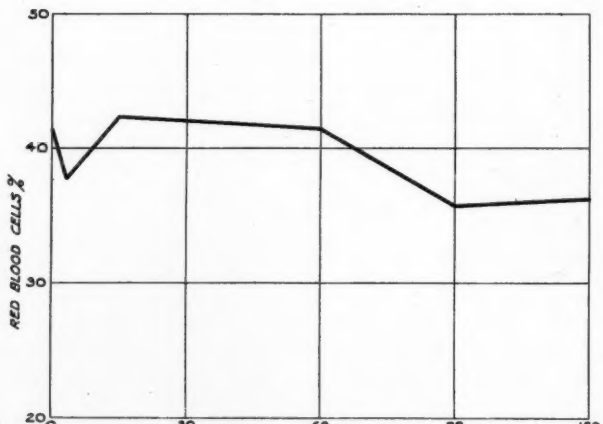
The average total excretion of chloride in 2 hours varied from 1·68 gm. in

the cases of high blood-pressure to 0·68 gm. in the chronic nephritics. In the normal cases the average was 1·33 gm.

The red cell volume curve shows a general similarity to the chloride curve. Immediately after the injection there is a fall in the proportion of cells (*i. e.*



CURVE 2.



CURVE 3.

the blood becomes more dilute). There is then a rise in 15 minutes nearly to the resting level (though sometimes there is a further fall at 15 minutes as with the minority of the chloride curves) and then usually a slight fall, with fluctuation so that in 2 hours the blood is usually slightly more dilute than before the injection, though sometimes it is slightly more concentrated.

TABLE I.—*Experimental Results in Six Cases selected at random.*

Case.	Time.	Blood.			Urine.			
		Urea (mgm. %).	NaCl (mgm. %).	Red cell volume (%).	Volume (c.c.)	Urea (%.)	NaCl (%).	Total urea excretion (gm.)
St— (normal) . .	Before	24	540	...	10	...	...	...
	3 mins.	44	517	...	(? bladder			
	15 „	35	533	...	not emptied)			
	1 hour	27	518	...	92	2·68	1·40	2·466 } 3·655
	1½ hours	26	506	...	...	...	...	... }
	2 „	...	...	...	47	2·9	1·60	1·189 }
G— (normal) . .	Before	26	540	45·7	180	1·8	1 5	...
	3 mins.	43	520	40·9	...	...	...	...
	15 „	33	540	37·8	...	...	...	...
	1 hour	32	520	...	46	2·5	1·8	1·15
	1½ hours	26	480	41·6	...	...	...	...
	2 „	28	460	41·9	?	2·4	1·4	...
D—(renal infantilism)	Before	216	612	...	155	·8	·32	...
	3 mins.	252	572	33	...	...	...	..
	15 „	230	644	29	...	...	...	...
	1 hour	229	606	38	143	·9	·28	1·287 } 2·187
	1½ hours	216	640	..	...	...	..	... }
	2 „	219	606	32·4	100	·9	·34	·9 }
D— (chronic nephritis)	Before	112	591	24·7	118	·6	·36	...
	3 mins.	122	583	20·8	...	...	...	...
	15 „	123	599	23·5	...	...	...	...
	1 hour	125	599	...	165	·5	·34	·825 } 1·364
	1½ hours	121	575	26·5	...	...	...	... }
	2 „	...	575	27·2	98	·55	·22	·539 }
P — (high blood-pressure)	Before	32	470	42	...	...	...	...
	3 mins.	57	470	48	...	...	...	...
	15 „	50	460	...	...	...	...	...
	1 hour	44	440	42	40	2·0	·46	·8 } 2·4
	1½ hours	36	470	47	...	...	...	... }
	2 „	...	460	46	66	2·4	·60	1·6 }
Sh— (hepatic cirrhosis with ascites)	Before	28	530	32·8	68	·6	·96	...
	3 mins.	45	510	32·2	...	...	...	...
	15 „	39	...	30·1	...	...	...	...
	1 hour	32	560	27 8	87	·9	·78	·783 } 1·098
	1½ hours	31	560	27·9	...	...	...	... }
	2 „	30	550	29·4	45	·7	...	·315 }

DISCUSSION.

(a) *The distribution of urea after the injection.*—A specimen of blood was taken 3 minutes after the injection because it is generally believed that a substance injected intravenously takes 3 minutes to mix completely with the circulating blood, though Lamson and Rosenthal (1922) state that in 3 minutes the injected substance is still unevenly distributed. In a few attempts to

settle this point it was found that the peak of the blood-urea curve occurred 3 to 5 minutes after the injection, and therefore the first specimen after the injection was taken then.

If the blood volume is taken at 7% of the body-weight, then in a man of 70 kilos. the blood volume would be about 5 litres. If all the 5 gm. of urea injected remained in the blood, the blood urea would be raised by 100 mgm. %. Actually the rise was 6 to 48 mgm. % in 3 minutes. If allowance is made for the body-weight (see Table II) it is found that except in one case, in 3 minutes only 10 to 28% of the injected urea remained in the blood. In the exceptional case (one of chronic nephritis with great œdema and ascites), apparently 40% of the injected urea remained in the blood, but since here the patient's weight included that of her œdema and ascitic fluids, the blood volume was almost certainly over-estimated, and it is therefore probable that actually less than 40% of the urea remained in the blood. The other chronic nephritics showed practically as rapid a passage of the urea out of the blood as the normals.

It seems probable, although the blood urea is usually still a little raised even after 2 hours, that diffusion is complete in 15 minutes. At this time, judging from the amount excreted in 1 hour, only 0·3 to 0·4 gm. of urea has been excreted. Now the amount of water in the body has been variously estimated. Volkmann (quoted by Elwyn) estimates it at 66% of the body-weight, while Mayer and Schaeffer (1913) say that the amount varies from 70% in the liver to 86% in the testicle. In addition there is the free water of the blood lymph and serous fluids. Against this, the amount in bone is small, and Marshall and Davis (1914) have shown that very little urea diffuses into fat. Probably the estimate that 75% of the body consists of water into which urea can diffuse is not far wrong. Thus in a 70 kilo. man there would be about 50 litres of available water. If the 5 gm. of injected urea diffused evenly into all this intra- and extra-cellular water, the concentration in the blood would be raised by 10 mgm. %. The table shows that the observed results agree fairly well with the expected figures, the body-weight being taken into account in the calculations. In order to prove that the urea diffused evenly into all the tissues it would be necessary to estimate the urea content of the tissues before and at intervals after the injection, which obviously is impossible in men. But in a few experiments the blood was centrifugalized immediately after it was withdrawn, and the plasma urea and cell urea estimated separately. Three minutes after the injection the plasma urea had risen by 11 to 20 mgm. %, while the cell urea had risen by only 5 to 14 mgm. %. In 15 minutes the urea content of the plasma and cells was practically identical.

Blood-urea curves after the intravenous injection of urea have also been investigated by Lamy and Mayer (1906), Leiter (1921), Marshall and Davis (1914) and Nonnenbruch (1921). Calculations from figures provided in their papers all agree in showing that immediately after the injection only 6 to 22% of the injected urea remained in the blood, thus agreeing well with our results. Marshall and Davis's figures for the amounts of urea in the tissues at varying periods after the injection also show that the urea had diffused evenly into all the tissue water, if corrections are made according to the figures supplied by

TABLE II.—*Calculations from the same Six Cases showing (1) Percentage of Injected Urea remaining in Blood Three Minutes after Injection; (2) Comparison of Actual Rise in Blood-Urea with Expected Rise if Urea had Diffused into all the Body-Water; (3) Effect of Injection on Distribution of Water and Chloride.*

Case.	Body-weight (kilos.).	Gm. of urea injected.	Time.	Actual rise in blood urea (mgm. %).	Percentage of injected urea remaining in blood.	Calculated rise in blood urea on basis of even diffusion (mgm. %).	Change in blood volume calculated from changes in blood and tissue urea (c.c.).	Actual change in blood chlorides (mgm. %).	Change in blood chlorides to be expected from calculated change in blood volume (mgm %).	Corrected calculation for change in blood volume, allowing for change in chlorides as well as urea (c.c.).
St— (normal) . . .	71	5·0	3 mins.	20	20	...	+44	−23	−5	−56
			15 „	11	...	9	+15	−7	−2	−13
			1 hour	3	...	5	...	...	...	...
			1½ hours	2	...	3·7	...	...	...	...
G— (normal) . . .	70	4·9	3 mins.	17	17	...	+25	−20	−3	−76
			15 „	7	...	9·2	−3	...	...	−3
			1 hour	6	...	7·4	...	...	...	...
			1½ hours	0	...	6·0	...	...	...	...
			2 „	2	...	4·6	...	...	...	...
D— (renal infantilism) .	36·3	5·0	3 mins.	36	18	...	+33	−40	−8	−55
			15 „	14	...	16·1	−3	+32	−1	−84
			1 hour	13	...	13·3	...	...	...	...
			1½ hours	0	...	10·9	...	...	...	...
			2 „	3	...	9·0	...	...	...	...
D— (chronic nephritis) .	81	4·9	3 mins.	10	19	...	+10	−8	−1	−62
			15 „	11	...	8	+115	+8	+2	+87
			1 hour	12	...	7	...	...	...	...
			1½ hours	9	...	6·6	...	...	...	...
P— (high blood-pressure)	53·7	4·8	3 mins.	25	20	...	+29	0	−4	+29
			15 „	18	...	10·6	+21	−10	−3	−21
			1 hour	12	...	9·2	...	...	...	...
			1½ hours	4	...	7·2	...	...	...	...
Sh— (hepatic cirrhosis with ascites)	61	4·8	3 mins.	17	15	...	+19	−20	−2	−76
			15 „	11	...	10·4	...	...	...	...
			1 hour	4	...	9·2	...	...	...	...
			1½ hours	3	...	8·4	...	...	...	...
			2 „	2	...	7·6	...	...	...	...

Note.—If the whole 5 gm. of urea injected had remained in the blood the expected rise in blood volume would be 311 c.c.

Mayer and Schaeffer for the amount of water in the various tissues. The results of the other observers mentioned, however, show that 15 or more minutes after the injection the observed blood urea was always less than that calculated on the basis of even diffusion into the body water. However, Lamy and Mayer's experiments were made in 1906, before blood-urea estimations were very accurate; with Leiter's results it was impossible, in making the calculations, to make an allowance for the urea excreted in the urine, so that the calculated figure was too high; while Nonnenbruch's results were so irregular as to raise doubts as to their accuracy. Again, Madsen (1924) found a higher concentration of urea in the tissues (except fat) than in the blood, and Rypins (1922) found that the concentration in various exudates and transudates varied from 37·5 to 180% of that in the blood. But it should be remembered that even if, for example, ascitic fluid has at the moment of its formation the same urea concentration as the blood, it immediately becomes mixed with previously formed ascitic fluid with perhaps a different urea concentration, and the resultant mixture might well have a different urea concentration to the blood, especially if rapid changes in the blood urea were occurring. There is universal agreement (see Folin and Berglund (1922), Barat and Hetenyi (1922), Plass (1923), Denis and Minot (1917) and others) that other nitrogenous bodies are not evenly distributed between the blood and tissues nor between the plasma and red cells.

The fact that the blood urea 15 minutes after the injection is at the level which might be expected if even diffusion into all the body had occurred, is certainly not in itself sure evidence that such a diffusion actually had occurred. Thus Folin, Berglund and Derrick (1924) found blood uric acid concentrations after the intravenous injection of uric acid agreeing closely with those to be expected if even diffusion had occurred; yet analyses of the tissues showed that actually most of the injected uric acid had been destroyed, and most of the tissues contained much less than the blood. Again van Slyke and Meyer (1913) found that after the injection of amino-acids the rise in the blood amino-nitrogen was sometimes that to be expected if even diffusion had occurred, while tissue analyses showed that actually the distribution was uneven. The correspondence between the observed and the calculated figures was accidental, and occurred in only a few experiments. Nevertheless, the constancy of our results, our observations on the partition of urea between plasma and corpuscles and the work of Marshall and Davis tend to show that in the case of urea even diffusion into all the body water does occur. No significant difference in this respect was found in the cases of chronic nephritis with high blood urea.

(b) *The effect on urea metabolism.*—In the normal subjects the average excretion of urea in the 2 hours of the test was 2·68 gm. In 16 normal people to whom no urea was given the average urea excretion in 2 hours was 2·45 gm. If urea formation goes on unchecked after the injection, the urea excreted is almost entirely accounted for by that formed in metabolism, and our conclusions as to the even diffusion of urea are vitiated, since in the calculations account was taken of the urea excretion. The fact that a further fall in the blood urea occurs after 15 minutes (when diffusion is complete) seems to have three possible explanations: (1) That the injected urea is destroyed; (2) that the

sudden introduction of urea into the blood checks the formation of urea in metabolism; (3) that, in spite of the evidence adduced, and which we are unwilling to reject, the concentration of urea in the tissues is greater than that in the blood, and that therefore the process by which the urea enters the tissues cannot be one of simple diffusion. If either of the first two explanations are correct it might be thought that the urinary excretion of ammonia would be increased after the injection. It was found that the urinary ammonia usually was increased, *e.g.* from 0·055 gm. in the hour before the injection to 0·106 gm. in the hour after. The ammonia content of the urea solution injected was 0·01 gm. It is evident that the degree of increased ammonia excretion (at the most 0·2 gm., representing about 0·36 gm. of urea, in 2 hours) is quite insufficient to account for the injected urea. It was nevertheless decided to follow the matter further by observing the effect of giving 15 gm. of urea by mouth on the ammonia excretion. It soon became evident that the acidity of the urine was the chief factor governing the ammonia excretion. If the urine was made alkaline by large doses of sodium bicarbonate, the ammonia excretion fell to a very low level, and was not materially increased by the administration of urea. Nash and Benedict (1921) think that the kidneys are the only organs which form ammonia, probably from urea. This is supported by Russell (1923), while Bliss thinks that other organs are also concerned. If only the kidneys formed ammonia it would be unlikely that during the 2 hours of the test there would be much increase in ammonia formation, since during this time most of the injected urea is diffused evenly over the whole body, and comparatively little reaches the kidneys. Wakeman and Dakin (1911) perfused surviving dogs' livers with saline containing up to 400 mgm. % of urea and found no increase in ammonia formation. Barnett and Addis (1917) found that after the intravenous injection of 15 gm. of urea into rabbits of 1 kilo, the blood ammonia rose from 0 to 9 mgm. %. Some of this was formed by bacterial decomposition of the urea in the bowel; when the bowel was excluded the maximum rise was 2·5 mgm. %. The injection of 15 gm. of urea into a rabbit of 1 kilo would raise the urea content of blood and tissues, after complete diffusion, by 2000 mgm. %. A rise of even 9 in the blood ammonia, representing 16 mgm. % of urea, is insignificant. On the other hand, Marshall and Davis have shown, by estimation of the urea content of blood, urine and tissues in a dog of 6·8 kilos after the intravenous injection of 20 gm. of urea, that in the first 24 hours after the injection 1·9 gm. of urea less than normal is formed in metabolism; the next day 2·2 gm. more than normal is formed, and the third day the normal amount is formed. It thus seems that in the 24 hours after the injection there is a destruction or diminished formation of urea, even if this does not show itself by an increased formation of ammonia, and that this is followed by an increased formation, perhaps from retained urea precursors. It is conceivable that in the first 2 hours after the injection, when the sudden shock of the injection is at its height, the formation of urea might practically cease. This would explain why in our experiments only 0·23 gm. of urea more was excreted than in the controls. This concept seems to have a bearing on the pathogenesis of uræmia. If the accumulation of urea in the blood and tissues results, according to the law of mass action, in a slowing of the formation of urea, then not only will urea precursors, which may or may

not be toxic in themselves, accumulate, but also those metabolic processes, the end stage of which is the formation of urea, will be slowed, and will ultimately cease, so that the patient dies. Some support for this is found in the work of Witts (1929), who found that the injection of 50 gm. of amino-acid caused less than the expected production of urea in uræmics.

(c) *The effect on the distribution of salt and water.*—The average initial level of the whole blood chlorides was 515 mgm. % in the normal cases, 485 mgm. % in the acute nephritics, 565 mgm. % in the cases of hepatic cirrhosis, and 595 mgm. % in the chronic nephritics. The plasma formed about 57 % of the blood except in the cases of cirrhosis of the liver, where the average was 70·5 %, and in the chronic nephritics, where it was 74·5 %. It is well known that the concentration of chloride in the plasma is about twice that in the corpuscles, and Siebeck (1919) has shown that this proportion is maintained however much the blood is diluted. It is thus to be expected that the whole blood chlorides would rise with increasing relative proportions of plasma, and Gram (1924), Haden (1924) and Cameron (1927) have demonstrated the expected rise in cases of anæmia. It is suggested that the high initial level of the blood chlorides in the cases of hepatic cirrhosis and chronic nephritis is due, in part at least, to this cause.

In the first 3 minutes after the injection of urea there is usually a sharp fall in the blood chlorides and in the red corpuscle volume, followed by a rise of both nearly to the resting level in 15 minutes, when the diffusion of urea is complete. Both curves then show a slight fall, or they remain almost constant near the resting level. The obvious inference is that water first enters the blood owing to the osmotic attraction of the injected urea, and then leaves it, as, in 15 minutes, the concentration of urea in blood and tissues becomes equal; and further, that the changes in the blood chlorides are dilution effects. But further examination of the data shows that this can only be a partial explanation (see Table II). A 1·61% solution of urea is isotonic with the blood. Calculations made from the amount of the injected urea remaining in the blood, together with that which had passed into the tissues (and there counteracted the osmotic attraction of that still in the blood), showed that the average expected rise in the blood volume, if the rise were due entirely to the osmotic attraction of the urea, was 28 c.c. (extremes, 0 to 59 c.c.) in 3 minutes, and 4 c.c. (extremes, — 23 to + 22 c.c) in 15 minutes. We have, of course, in the red cell volume curve data which might be expected to show the variations in the blood volume, but, as already stated, there are so many fallacies in this method of estimating the blood volume that we regard the results as of value only qualitatively, not quantitatively, and this is borne out by the very variable results obtained from these data, which are obviously useless in determining whether or not the dilution of the blood is due to the osmotic attraction of the urea remaining in the blood.

The average observed depression of the blood chlorides below the resting level was 15 mgm. % in 3 minutes and 18 mgm. % in 15 minutes. If the fall in blood chlorides were a dilution effect, and if the degree of dilution were that calculated from the blood and tissue urea concentration, then the average depression of chlorides in 3 minutes would be 4 mgm. %, and in 15 minutes 1 mgm. %. The observed fall in blood chlorides is therefore greater than can

be accounted for by the calculated dilution of the blood. It thus seems that chloride is actually leaving the blood in the first 15 minutes after the injection. If this factor is taken into account in a fresh calculation of the osmotic changes in the blood, it would be expected that the average blood volume would actually fall by 37 c.c. in 3 minutes, and by 89 c.c. in 15 minutes. It thus seems that more salt leaves the blood than is necessary to counteract the rise in osmotic pressure due to the presence of an excess of urea in the blood, so that if osmotic pressure alone were responsible for the blood-volume changes, the blood volume would fall. Nevertheless, the shape of the red-cell volume curve is so constant, and so clearly indicates a dilution of the blood in 3 minutes in practically every case, that we are unwilling to believe that the curve is of no value at all. The contradiction might possibly be explained if it be assumed that the movement of water lags behind that of urea and salt, so that the dilution found at 3 minutes represents the osmotic state of the blood a little before, when little urea or salt had left the blood.

At the end of 15 minutes the urea has diffused completely into the tissues, and the rise in osmotic pressure which presumably initiated the movement of the salt out of the blood has disappeared. The salt, therefore, usually tends to re-enter the blood. But the fact that it rarely quite reaches its resting level, and that often a further fall instead of a rise occurs in the 15 minutes, indicates that another factor is at work. It is suggested that some of the chlorides pass out of the body fluids to be stored in a "dry" form, possibly in some sort of combination with protein, in the salt depots, which have been shown to exist in the skin by Padtberg (1910) and Wahlgren (1909). This would tend to restore the crystalloid balance of the body-fluids, which had been disturbed by the introduction of a large quantity of urea. The following is a specimen of the calculations on which this is based:

In a case of infective endocarditis, the calculated blood volume before the injection was 5·3 litres.

The red-cells formed 28% of the blood before, and 26·5% of the blood 2 hours after, the injection.

Therefore blood volume after injection was 5·6 litres.

Blood chlorides before injection were 575 mgm. %, therefore total chloride content of blood before injection was 30·48 gm.

Blood chlorides after injection were 536 mgm. %, therefore total chloride content of blood after injection was 30·02 gm.

Therefore blood loses 0·46 gm. chloride.

Loss of chloride in urine in 2 hours was 0·67 gm.

Therefore tissues lose 0·67 − 0·46, or 0·21 gm. of chloride.

Now tissues lose 300 c.c. of water to the blood,
154 c.c. ,, in the urine,
and (say) 76 c.c. ,, ,, breath, sweat, etc.

Therefore tissues lose 530 c.c. of water and 0·21 gm. of salt.

Now if the tissue fluids are in equilibrium with the blood, they will contain 536 mgm. % of salt. If the 530 c.c. of water lost by tissues carried all its salt with it, the tissues would have lost 2·89 gm. salt instead of 0·21 gm. Therefore the tissues, as distinct from tissue-fluids, are more concentrated in salt to the extent of 2·68 gm., and most of this is probably stored in the salt depots. It is noteworthy that at this stage 2·51 gm. of the injected urea still remains in the body, and this seems to have displaced 2·68 gm. of salt.

There are four possible fallacies in this calculation. First, the loss of water in the sweat, etc., has been estimated at 76 c.c. on the basis of Veil's

not be toxic in themselves, accumulate, but also those metabolic processes, the end stage of which is the formation of urea, will be slowed, and will ultimately cease, so that the patient dies. Some support for this is found in the work of Witts (1929), who found that the injection of 50 gm. of amino-acid caused less than the expected production of urea in uræmics.

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There are four possible fallacies in this calculation. First, the loss of water in the sweat, etc., has been estimated at 76 c.c. on the basis of Veil's

figures (1913) that the extra-renal water loss is 30–50% of the renal. There is no evidence as to the amount lost in this case, but probably the estimate of 76 c.c. is not far wrong. Secondly, the body fluids have been taken as being in equilibrium with the blood instead of with the plasma. In the case quoted, instead of containing 536 mgm. % of salt, they would contain 600 mgm. % or more, and the amount of salt retained would be greater than that calculated. Thirdly the red-cell volume is an inaccurate guide to the blood volume; but even if the blood volume changes are neglected, the calculations show that the tissues become more concentrated in salt to the extent of 3·17 gm. Fourthly, throughout the test an unknown amount of salt and water is absorbed from the alimentary tract. If the salt solution absorbed is less concentrated than the total of that lost in the urine, breath, etc. (*i. e.* less concentrated than 0·67 gm. in about 250 c.c.), the calculations are vitiated; but if, as seems much more likely, the concentration of the salt solution absorbed from the intestine equals or exceeds that lost from the body, then the conclusions reached hold good.

Calculations like these were made in 15 cases. In 7 they indicated that 0·16 to 4·0 gm. of salt were stored in a "dry" state. In 3 cases the findings were equivocal, the results varying as allowance was or was not made for alterations in blood volume. In 5 the tissues seemed to become more dilute in salt. It is noteworthy that in all these 5 œdema was, or had recently been, present. Two were cases of hepatic cirrhosis with ascites, 1 of heart failure, and 2 of acute nephritis. In only one of the other cases, one of chronic nephritis, was there any œdema. It is therefore suggested that the diffusion of the injected urea into the tissues in non-œdematous cases causes an increase in the amount of salt stored in a concentrated form in the salt depots of the body, perhaps in an attempt to reduce the crystalloid content of the body fluids which had been raised by the introduction of the urea. In œdematous cases the opposite result is produced. Possibly the elucidation of the nature of this different relationship to salt and water might do much to solve the obscure problem of œdema.

(d) *Comparison of the effects of urea after intravenous injection with that of other substances.*—Since calcium is reputed to be of great importance in regulating the permeability of cell membranes (Hamburger, 1922), it was decided to repeat the experiments using a 10% solution of calcium chloride instead of urea; but since the injection produced very unpleasant effects in ourselves, we did not feel justified in repeating the experiment on others. In our two experiments it was found that in 3 minutes 35 and 31% of the injected calcium was still present in the blood, as against 10 to 28% in the case of urea, and that in 15 minutes the serum calcium was 0·82 and 1·37 mgm. % above the resting level, instead of 0·19 and 0·36 mgm. % respectively, as would be required if the calcium had diffused evenly into all the body water. At the end of 1½ hours the serum calcium was 0·09 mgm. % above and 0·39 mgm. % below the resting level respectively. Since the calcium excretion was not estimated, the meaning of this is not clear, but it suggests storage of the calcium in a concentrated form in the calcium depots (the cancellous tissue of bones). These results are borne out by experiments of Walters and Bowler (1924) on dogs. They found that in normal dogs 14–36% of the injected

calcium was still present in the blood 5 minutes after the injection, and that it had all disappeared in ½–1 hour, which certainly does not leave adequate time for excretion. In jaundiced dogs, however, they found that calcium left the blood more quickly and completely than urea.

Experiments with sodium chloride have been made by Barach *et al.* (1922), Denis (1923), Hamburger (1891), Lamy and Mayer (1906), Schenk (1920), Schmid and Schlayer (1911) and Weber (1911), usually on dogs. Calculations from their results show that sometimes more, sometimes less chloride leaves the blood after the injection than is necessary to equalize the salt concentration in blood and tissues, but in no case are data given to enable a calculation to be made as to the proportion of salt leaving the blood in 3 minutes. There is much evidence that after the injection the concentration of salt in some tissues is greater and in others is less than in the blood. Padtberg has shown that after the injection as much as 75% of the salt remaining in the body may be stored in the skin. Wahlgren found in dogs the highest concentration of salt in the skin and the lowest in muscle. After the injection of salt the skin still contained the highest and the muscles the lowest concentration of salt, but owing to the great bulk of the muscles most of the injected salt was stored in them. It was noteworthy that the movement of water did not run parallel with that of salt. Indeed the skin and muscles lost water in spite of the increased salt concentration.

With sodium sulphate (Denis and Minot, Greenwald, Hamburger) more than the expected amount of sulphur remains in the blood, while this is only sometimes true of the sodium ; in other cases more sodium enters the tissues than is necessary to bring them into equilibrium with the blood. Similarly more magnesium, phosphate ion and bicarbonate (Reimann and Reimann, 1921) pass into the tissues than is necessary to make the concentration there equal to that in the blood. Lactose (Schwartz and Pulay, 1915) does not readily leave the blood. The same is sometimes true of glucose, while in other cases more leaves the blood than is necessary to bring the tissues into equilibrium with it. Palmer (1917) has shown that the distribution of glucose between the blood and the various tissues in normal dogs is very irregular.

Some dyes, such, as trypan red (Seyderhelm, 1923) and vital red (Hooper, 1920), leave the blood very slowly. Phenoltetrachlorphthalein (Rosenthal, 1922), pass out even more rapidly than does urea, but the evidence shows that it is largely removed by the liver. Phenolsulphonephthalein leaves the blood about as quickly as urea (Dawson, 1920), but much more of it is excreted in the urine, and, as Putnam (1922) has shown, much less passes into the peritoneal cavity, so that it is probable that an unduly large proportion is separated off by the kidneys. Starling (1893) has shown that peptone reaches a higher concentration in the lymph than in the blood after intravenous injection. Gelatin (Boycott, 1913), olive oil (Busquet, 1920), optochin (Schnabel, 1920), strophanthin (Gottlieb, 1917) and arsphenamine (Kolls and Youmans, 1923) all leave the blood more slowly than does urea, and the distribution of the last in the tissues is very irregular, some, such as the liver, containing a much higher concentration than the blood, others, such as the brain, a much lower concentration.

It thus seems that urea is unique among the substances examined in that

it diffuses evenly into all the water of the body. With the other substances, some tissues contain the injected substance in greater, others in less concentration than the blood, indicating that the process by which these substances leave the blood is not simple diffusion alone.

The literature also gives data for calculating the effects of the injection of various substances on the distribution of various constituents of the blood. Iso- and hypotonic saline or Ringer's solution rapidly leaves the blood after injection, so that the greater part has entered the tissues before the injection is complete. Very strong saline remains longer in the blood, and may even attract more water from the tissues. Weed and McKibben (1919) and Foley and Putnam (1920) have shown that after the injection of strong saline the cerebro-spinal fluid pressure falls and the brain shrinks. Hamburger (1891) has shown that the injection of large amounts of hypertonic saline causes only slight and transient changes in the osmotic pressure of the blood-serum, a fall in the concentration of serum protein and bicarbonate neutralizing the rise in osmotic pressure due to the excess of salt. Calculations from his figures make it probable that the fall in concentration of serum protein and bicarbonate is not due entirely to dilution, but that some of each actually leaves the blood (though perhaps the differences between the calculated and observed figures are not great enough to warrant this conclusion). He also show that the strength of saltpetre solution which just causes hæmolysis is practically unaltered after the injection. Since salt enters the red cells, this means that other substances leave them. Greenwald (1918) found that after the injection of an amount of sodium sulphate and phosphate about equal to the blood volume, the serum calcium was halved. Since it is incredible that all the injected fluid remained in the blood, it seems that calcium must have left the blood. Calculations from Nonnenbruch's figures shows that after the injection of 10% urea solution in normal saline protein sometimes leaves, sometimes enters the blood. Bürger and Hagemann's (1920) figures give similar contradictory results after the injection of glucose. Magnus (1900) shows that a great loss of protein from the blood follows the injection of large quantities of saline; as much as 19% of the total serum protein may leave the blood during the infusion, while water is still leaving the blood, and a further quantity may be lost after the infusion has ceased, while water is re-entering the blood to be transported to the kidneys for excretion. Again, Lamy and Mayer have shown that the fall in blood chlorides after the injection of saccharose is greater than can be accounted for by dilution. On the other hand, the figures given by Lipschitz (1919) indicate that after the injection of glucose, salt and non-protein nitrogen enter the blood from the tissues.

Thus the results of some observers with the intravenous injection of various substances suggest that the blood is merely diluted by the water injected, or by that entering the blood owing to the osmotic attraction of the substance injected. The results of others suggest that the water entering the blood from the tissues carries with it dissolved substances in low concentration, so that while the concentration of these substances in the blood is reduced, the total quantity is increased. A third group of observations suggests that other substances, as well as that injected, leave the blood after the injection, and that therefore the total quantity of these, as well as their concentration in the blood,

is reduced. It is noteworthy that colloids, such as plasma proteins, may pass out of the blood in this way—a fact of considerable importance in connection with the pathogenesis of œdema. Our own observations on the behaviour of the blood chlorides seem to show that after the injection of urea, chlorides as well as urea pass out of the blood. It seems to us more likely that when the concentration of one crystalloid in the blood is suddenly increased, all the other crystalloids, and perhaps even some of the colloids, will tend to leave the blood and pass into the tissues in an attempt to restore equilibrium.

(e) *Factors influencing the interchange between blood and tissues.*—The capillaries are almost completely permeable to water and to most of the substances dissolved in the blood-plasma. It is thus difficult to understand why the plasma does not ignore the bounds of the capillaries and pass into the tissues, as it would if it were circulated through tubes of filter-paper (representing the capillaries) suspended in a bath half full of serum (representing the tissues). Obviously under these conditions the circulation could only be maintained after the tissues were saturated with water. Since when a large amount of fluid is injected intravenously it all rapidly passes into the tissues, it is obvious that the tissues are not saturated. The thesis of Elwyn's recent book on 'Edema' (1929) is that the volume of the blood is kept constant by the action of a centre, possibly in the hypothalamus, which regulates the permeability of the capillaries (including those of the renal glomeruli, thus regulating the urinary flow), the water capacity of the tissues, etc. Possibly this is a factor to be considered, but it seems much over-emphasized.

Since salt and other crystalloids readily pass through the capillary walls they are unable to exert osmotic pressure while inside the capillaries, and therefore they are not responsible for keeping the plasma inside the vessels. Moreover there is much evidence that salt can be stored in the tissues in a more concentrated form than in the blood, and that it does not then attract water. But Priestley and Haldane (1915, 1921) have shown that salt may temporarily hold water in the body, the diuresis produced by drinking large quantities of saline being less intense and more prolonged than that produced by drinking the same quantity of water.

Starling emphasizes the importance of the osmotic pressure of the plasma proteins in balancing the capillary blood-pressure and so retaining the plasma in the vessels. He estimated both the colloid osmotic pressure and the capillary blood-pressure at 30 mm. of mercury. Schade and Claussen (1924) support this view, but show that the colloid osmotic pressure is really only about 24 mm. of Hg. Fahr and Swanson (1926) state that a further correction has to be made owing to the Donnan effect, in virtue of which the concentration of salt and other substances to which the capillaries are permeable is higher on the tissue side of the capillaries. This brings the "effective" colloid osmotic pressure down to about 20 mm. of Hg. A further reduction must be made as the capillaries are not completely impermeable to protein, as is shown by the fact that the lymph contains up to 4% of protein (the plasma contains 8%), and by the experiments of Magnus, which show with what ease large amounts of protein leave the blood after the injection of saline. All this means that the efficacy of the colloid osmotic pressure in maintaining the blood in the vessels is less than is usually supposed, and it is doubtful whether by itself it is

enough. Further support for this will be found in the section on œdema. The water-holding power of the tissues may also influence the exchange between blood and tissues, but little attention has been paid to this. It is obvious that if at any moment the tissues are saturated with water, no fluid will leave the blood even if conditions are otherwise favourable. If it is imagined that the intravenous injection of water in some way increases the affinity of the tissues for water, then the added water would leave the blood till the tissues were again saturated. Obviously the introduction of water into the blood would help the exit of fluid by reducing the colloid osmotic pressure and perhaps also by raising the capillary blood-pressure, but it is equally obvious that this would not be enough so long as the tissues were saturated with water. Martin Fischer has shown that very slight changes towards the acid side in the reaction of the tissues greatly increases their water-holding power. It is conceivable that rapid changes in the acid-base balance might occur after the injection of water, but there is no evidence as to this. Again, it is well known that changes in the relative concentrations of Na, K and Ca ions influence the rate of passage of water through animal membranes, and also, as shown by Martin Fischer, the degree of imbibition of water by proteins. Thus Chiari and Januschke (1911) show that calcium salts diminish or prevent experimental œdema. Hamburger (1922) shows that œdema occurs in a perfused frog's leg unless the perfusion fluid contains an adequate proportion of calcium. Smith and Mendel (1920) show that the addition of calcium to saline injected intravenously increases the rate at which fluid leaves the blood, thus suggesting that the œdema-preventing action of calcium is due to its increasing the water-holding power of the tissues and so preventing the appearance of free fluid in the tissue spaces. On the other hand, calcium causes a diuresis in some cases of hydræmic nephritis, and Hülse suggests that it reduces the water-holding power of the tissues, so that water is given up by the tissues to the blood, to be excreted by the kidneys. This explanation is supported by Martin Fischer. The evidence on this point is therefore contradictory.

Thus it seems that of the factors considered, the colloid osmotic pressure of the plasma and perhaps the limitation of the water-holding power of the tissues are most concerned in keeping the plasma in the vessels, but it is possible that some other unconsidered factor may prove of much more importance than either.

(f) *Œdema.*—This can only be considered in so far as this work touches upon it. None of the many explanations of the production of œdema seems satisfactory. Even in the case of cardiac œdema, apparently easily explainable on the grounds of excess of capillary blood-pressure over colloid osmotic pressure, other factors seem to be at work. Thus it has been shown that in animals, ligation of the femoral vein is not enough to cause œdema. It has been suggested that in cardiac œdema the capillaries are damaged and their permeability increased by the deficient oxygen supply. But increased capillary permeability can only act by increasing the filtering force relative to the resistance to filtration, and it has yet to be shown that any increase in the filtering force is adequate to produce œdema. Again, any increase in capillary permeability would cause an increased protein concentration in the œdema

fluid; but the protein content of the œdema fluid in heart failure rarely much exceeds 1%, while normal lymph contains as much as 4%. Only in inflammatory œdema does there seem to be any evidence of increased capillary permeability. Indeed there seems to be very little evidence that capillary damage does cause increased permeability. Thus in capillary damage by uranium the capillary permeability seems to be diminished.

The œdema of "lipoid nephrosis" is usually thought to be due to a diminished colloid osmotic pressure due to a reduced plasma protein concentration, and Christian (1929) states that in animals most of the symptoms of the disease could be produced by maintaining the plasma proteins at a low level. But a low colloid osmotic pressure is merely equivalent to a relative rise in the filtering pressure, and the arguments against this as a cause of œdema have already been stated. Moreover, several cases have been reported in which the œdema has disappeared while the colloid osmotic pressure remains as low as ever. Again, if a low colloid osmotic pressure increased the filtration of fluid through the capillaries it would be expected that there would be polyuria instead of oliguria as actually occurs, and the polyuria would be increased by the fact that, as shown by the albuminuria, the glomerular capillaries are permeable to protein, so that the effective colloid osmotic pressure would be still further reduced. Magnus, Oehme (1921) and others have shown that dilution of the blood and therefore reduction in the colloid osmotic pressure need not cause polyuria. These arguments seem to make it improbable that a low colloid osmotic pressure is the cause of œdema. This is supported by the arguments previously mentioned against the colloid osmotic pressure being the only cause of the retention of the plasma in the vessels.

The evidence already discussed indicates that salt has no permanent effect in altering the distribution of water. The diminished excretion of salt in nephrosis has been shown to be due to a retention of salt in the tissues, and not to inability of the kidneys to excrete it. This shows that in many cases of œdema the tissues have an undue affinity for salt, but which is cause and which effect it is impossible to say. Our experiments show that the reaction to the injection of urea is altered in œdema, in that salt is displaced from the depots instead of being deposited in them.

The experiments of Boycott, Chisholm (1913), Schwartz and Pulay (1915) and Pollak (1924) have an important bearing on the problem. Boycott showed that after ligature of the ureters, normal Ringer's solution left the blood more quickly and more completely in normal animals than in animals made nephritic by uranium. In spite of this, œdema never occurs in the normal animals, but is common after the injection in the nephritic. It thus seems that the tissues of normal animals are able to take up and bind large quantities of water, while in uranium nephritis this power is lessened, so the water entering the tissues remains free and visible as œdema fluid. Our observations on the behaviour of salt after the injection of urea suggest that in œdema the tissues are less able to bind salt as well as water. It seems that this factor must be reckoned with in the production of œdema, as well as an actual increase in the amount of water in the tissues, though there is evidence against as well as for it. Thus Aldrich and McLure show that the wheal raised by the intradermal injection of saline disappears less quickly in health

than when there is œdema or a tendency to œdema. But this does not necessarily mean that the fluid injected becomes bound to the tissues; it may merely mix with the œdema fluid.

SUMMARY AND CONCLUSIONS.

1. The results of the intravenous injection of 5 gm. of urea in health and in various diseases were investigated. By the time the urea has mixed with the blood (3 minutes) 80% has left the blood-stream. It it suggested that the urea leaves the blood by a process of simple diffusion, passing into the water of the cells as well as into the free water; and that the process is complete in 15 minutes, when the concentration of urea in the blood equals that in the tissues.

2. A review of the literature indicates that urea is unique in this respect. With no other substance investigated is the distribution uniform over blood and tissues, and in most, the concentration in some tissues is greater than in the blood, showing that the process by which the substance enters the tissues is not one of simple diffusion alone.

3. It is suggested that the sudden introduction of urea into the body delays the formation of urea, and may even bring it to a standstill for the first two hours after the injection. The importance of this in connection with the production of uræmia is mentioned.

4. The osmotic attraction of the urea injected is greatly reduced by its rapidly leaving the blood. Salt also leaves the blood, so that the osmotic attraction of dissolved substances in the blood is not raised even 3 minutes after the injection. In spite of this the blood is more dilute at the end of 3 minutes. It is suggested that this may be due to a lag in the movement of water behind that of urea and salt.

5. Evidence is discussed in favour of the view that except in œdematous patients urea injection causes some salt to pass out of the body fluids, to be stored in a "dry" form in the salt depots of the body, thus reducing the crystalloid concentration of the body fluids which had been raised by the injection of urea; but that in most cases of œdema, salt is liberated from the depots and enters the body fluids.

6. It is pointed out that the capillaries are completely permeable to water and to most of the substances dissolved in the blood plasma, and are partly permeable to the plasma proteins. The factors retaining the blood inside the vessels are discussed. None of the causes ordinarily mentioned seem sufficient. It seems probable that the true cause must be sought in the tissues, rather than in the blood or blood-vessels. It is suggested that perhaps the amount of fluid leaving the blood is limited by the capacity of the tissues for taking up water, and that this in its turn may be influenced by changes in the acid-base balance, or by alterations in the relative proportions of various ions.

7. A review of the literature shows that in experimental œdema usually less fluid passes into the tissues, after intravenous injection, than in non-œdematous animals, suggesting that an important factor in the production of œdema is a deficient power of the tissues to hold and bind water, which therefore accumulates in the tissue spaces as œdema fluid. Our own results suggest

that in œdema the tissues are also less able to bind salt, which also accumulates in the free tissue fluids. Any satisfactory theory of œdema must account for this.

We wish to express our indebtedness to the Physicians and Surgeons of the Queen's Hospital who allowed us to investigate their cases, and to Miss Hickman, M.Sc., Biochemist at the Children's Hospital, Birmingham, who very kindly undertook the calcium estimations in two of the cases.

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THE INTRA-OCULAR PRESSURE AND THE DRAINAGE OF THE AQUEOUS HUMOUR.

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Received for publication October 2nd, 1930.

A RECENT paper in this Journal on this subject (Ridley, 1930) calls for one experimental record and some comments. The author seeks to revive a very old hypothesis that the drainage of the intra-ocular fluids occurs, not through Schlemm's canal into the anterior ciliary veins, but through the cornea into the conjunctival sac.

After considering the anatomical relations at the canal of Schlemm in the light of the work of Henderson (1908) and Thomson (1911)—most of the questions which are raised are adequately answered in the classical work of Maggiore (1917)—Ridley concludes that " since minute particles such as those of Indian ink do not pass into the lumen of the canal from the anterior chamber (Nuel and Benoit, 1895), there is evidence that no such direct communication beween the anterior chamber and the canal exists. The failure of particulate matter, however, to pass through the endothelial cells of Schlemm's canal has no possible relation to the passage of fluid through them. Ridley has failed to show that particulate matter is delivered out of the eye through the cornea ; this argument, therefore, denies his main thesis.

The theory of the drainage of the aqueous humour through the cornea is based upon experiments which show :

(1) That the cornea of the dead eye acts as a permeable membrane under the influence of the osmotic pull of a solution of cane-sugar ; this applies to almost every tissue.

(2) That substances can diffuse from the conjunctival sac into the anterior chamber ; this has been accepted since the work of de Ruiter (1853), v. Graefe (1854) and others.

(3) That substances introduced into the anterior chamber diffuse through the cornea, entering at the site of the filtration angle ; this has been generally accepted since the work of Laqueur (1872), Gruber (1894) and Leber (1889–1903). It is to be remembered, however, as was observed by Parsons in discussing the same hypothesis in 1904, that the phenomena of the diffusion of foreign substance need bear no relation to the normal flow of fluid.

(4) That the rate of drainage through the cornea equals the rate of formation of the intra-ocular fluid. The first of these measurements was obtained by measuring the rate of flow under a pressure head of 20 cm. water through a dead cornea—a measurement which must have merely a hypothetical relation with the conditions in life. The second is taken from Friedenwald (1930), quoted as unpublished experiments, " wherein the time required for the evacuation of the aqueous could be measured." On this point my remarks (pp. 85 *et seq.*, 1927) still hold good : the conditions of experimentation on the evacuation of the aqueous humour are completely artificial and, involving a complete dislocation of the pressure equilibrium in the eye, can bear no relation to the normal conditions.

Against these experiments I would put forward one experiment only, which to my mind is conclusive :

Experiment.—A needle connected to a reservoir is inserted into the anterior chamber of an anæsthetized dog. The system is filled with methylene-blue and the pressure equilibrated by means of an air-bubble. The reservoir is raised, giving a pressure-head of 1 to 2 mm. Hg. Coloured fluid slowly flows into the anterior chamber ; in 1½ minutes the episcleral veins are deeply dyed. There is no evidence of any coloration of the cornea nor the minutest evidence of dye in the conjunctival sac. Admittedly the circulation here is an artificially induced pressure circulation, but the available channels of exit are obviously *viâ* Schlemm's canal and the episcleral veins and not by way of the cornea.

With regard to the mechanism of the intra-ocular pressure, Ridley shows

that the sclera is distensible, and thereby corroborates the work of Weber (1876), Ischreyt (1899), Koster (1895, 1900, 1903) on pig, rabbit and human eyes, but his conclusion that changes in the intra-ocular pressure are determined by changes in the vascular bed of the uveal tract to the exclusion of changes in volume of the ocular media does not conform to facts. No one denies that changes in the circulation influence the intra-ocular pressure profoundly, but I have shown (1926–29) that hypotension can occur with maximal vaso-dilatation. He denies a swelling of the vitreous [without producing any evidence to refute its proved occurrence [Duke-Elder, 1928]), and considers that the clinical appearance of an eye in glaucoma is due to a turgescence of the uveal vessels pushing the vitreous forward. Admittedly such a mechanism may exist—but not exclusively. Commenting on a similar suggestion put forward by Magitot (1917), I wrote (1927) that section of an eye with chronically raised tension frequently shows a thin and atrophied choroid, ciliary body and iris, the whole uveal tract being obviously pressed almost out of existence by something else (the vitreous).

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A STUDY OF THE BLOOD-PIGMENT IN OBSTRUCTIVE JAUNDICE, WITH OBSERVATIONS ON THE VAN DEN BERGH REACTION.

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Received for publication September 15th, 1930.

THE investigation of the blood-pigments in jaundice by Van den Bergh and his discovery of two types of reaction to Ehrlich's diazo-reagent, the "direct" and the "indirect," gave a fresh impetus to the study of this clinical condition. Van den Bergh's work has been well described by McNee (1923) and we are indebted to his paper for much of our information in regard to the work and conclusions of Van den Bergh.

The nature of the difference between the two types of pigment, as revealed by the diazo-reaction of Van den Bergh, presented an interesting problem. Van den Bergh regarded the pigment as bilirubin, and suggested that there was a slight chemical difference between the two forms, or perhaps a modification of the pigment by attachment to the protein of the serum; addition of alcohol, as in carrying out the indirect reaction, might then have the effect of breaking down the protein combination with the liberation of bilirubin. Andrewes (1924) was unable to find evidence of the existence of a combination of protein with bilirubin. Several other investigators have attacked this problem, with results which do not agree very well. It would appear that the principal point at issue is which type of reaction is given by bilirubin as ordinarily prepared from gall-stones or bile. But it is at this step that there is wide divergence of opinion. Some workers hold that bilirubin gives an indirect reaction in serum, and that it is an ammonium salt (Collinson and Fowweather, 1926) or an unknown combination of bilirubin (Roberts, 1928) which is responsible for the direct reaction. Thannhauser and Andersen (1921) have suggested that the pigment in sera giving the indirect reaction is not bilirubin. Others present evidence that serum containing bilirubin gives a direct reaction, whereas oxidation products of bilirubin (Davies and Dodds, 1927) or a difference in its chemical nature (Newman, 1928) characterizes indirect-reaction pigment.

In another communication (Griffiths and Kaye, 1930) we have stated that pure bilirubin does not give a prompt direct reaction when suspended in serum. Claims which have been made that it does so are, in our opinion, due to the fact that the samples of bilirubin used have been contaminated by a second pigment, also present in bile, which we showed was capable of giving the direct reaction when dissolved in serum. This second pigment remains when acidified bile, after drying *in vacuo*, is exhaustively extracted with

chloroform, using the precautions described by us. Briefly, two pigments occur in fresh bile: the first, bilirubin, is insoluble in water, soluble in chloroform and alcohol and is responsible for the indirect reaction in serum; the second, "direct reaction" pigment, is soluble in water and alcohol, only very sparingly soluble in chloroform, and couples with diazo-reagent in the absence of alcohol. This pigment gives the direct reaction in serum. "Direct-reaction" pigment is very much more unstable than bilirubin, and rapidly loses its power of giving a direct reaction, though retaining for a longer time the indirect reaction. This pigment does not appear to be one of the ordinary salts of bilirubin.

Solutions of "direct reaction" pigment in serum appear to have all the properties of direct-reaction serum, but in order to establish their identity, we have made direct comparisons of such solutions with sera from cases of obstructive jaundice, which comparisons go to form the subject of this paper.

LIGATION OF THE COMMON BILE-DUCT OF THE RABBIT.

In a series of seven rabbits the common bile-duct was ligated close to the duodenum under ether anæsthesia. One animal succumbed, apparently from hepatic necrosis and extensive hæmaturia. The remaining six recovered well from the operative shock, and all developed icterus. Food and water were given *ad lib.* after operation.

The animals became very consistently jaundiced; the icterus was never more than suggested in the conjunctiva, but examination of the blood-serum and of the urine left no doubt of its early and definite appearance. The van den Bergh reaction of the serum of these animals, 24 hours after operation, was in one case negative; in two, direct but faint; in the remainder, prompt and of good intensity. Forty-eight hours after operation the serum of all the animals was definitely icteric, and the reaction prompt, direct and marked. The colour was in every case red, with a tendency to assume a more violet hue on standing. The appearance of the initial red reaction was never delayed for more than a few seconds after addition of reagent. The test was effected by adding an equal quantity of the diazo reagent to the serum.

Lepehne (1921), working with rabbits, found a prompt direct reaction in the serum of three of his animals within 24 hours of operation; one of these reactions was of the "biphasic" type, *i. e.* red changing to violet-red, as we found. A fourth animal yielded a "biphasic delayed" reaction only after about 50 hours, and a fifth after about 72 hours. Davies and Dodds (1927) quote one experiment in which a faint direct reaction appeared in 25 hours, and a true direct in 66 hours.

One of our animals was killed after 24 hours, another after 48 hours; three were allowed to go on for 72 hours, when they showed signs of sickness, with loss of weight and refusal of food. One animal remained fairly well for 125 hours. All the animals passed large quantities of highly pigmented urine. Approximately coincident with the appearance of a direct reaction in the blood-serum, the urine gave a red colour on addition of diazo-reagent. Traces only of bile-salts could be detected in the urine.

Post-mortem the gall-bladders and bile-ducts were found to be greatly

dilated with dark green bile, which was non-reactive towards diazo-reagent. With one exception, in which the liver was much enlarged and pale with a "nutmeg" appearance, the livers were small and abnormally dark and showed very marked biliary congestion. Microscopically, "the bile-channels were seen to be full of dammed-up bile; jaundice had in some cases spread out into the liver-cells. There was acute and chronic cholangitis of the large and small bile-ducts, and in one instance fibrosis had started, and was spreading out from the bile-ducts at the portal angles."

Examination of the blood-serum from these animals when icterus was well established showed that the pigment was, in the main, insoluble in chloroform after acidification. In two instances a small amount of the pigment was found to be soluble in chloroform. This chloroform-soluble pigment was presumably bilirubin, but its removal by chloroform left in the serum the insoluble pigment, which was very prone to turn green and to lose its diazo-reaction after exposure to air. If the reagent were added promptly after removal of the chloroform-soluble fraction, it was usually possible to observe an enfeebled but definite red-purple diazo-reaction in the serum. The simultaneous occurrence of chloroform-soluble and chloroform-insoluble pigments has been noticed by us in some human icteric sera; here, too, a purple diazo-reaction was given by the aqueous fraction after acidification and removal of the chloroform-soluble pigment. The pigment of the rabbit serum was observed to be very largely adsorbed on to the protein precipitate on addition of alcohol to the serum.

Using the serum of these animals, and also some samples of direct-reaction serum from human subjects, we carried out a series of tests, comparing the properties of direct-reaction serum with those of a solution of "direct-reaction" pigment (from bile) in normal serum. pH determinations were made with the glass electrode (Kerridge, 1925).

THE EFFECT OF ACIDIFICATION OF DIRECT-REACTION SERUM.

Serum from a case of gall-stone jaundice, having a prompt direct diazo-reaction, and serum from rabbits with experimental obstructive jaundice 72 hours after operation, were compared as follows: A series of test-tubes was set up, each tube containing 0·5 c.c. of serum from the case of gall-stones. Varying amounts of N/2 hydrochloric acid, together with sufficient water to compensate for dilution, were added. Finally, to each tube 0·5 c.c. of van den Bergh reagent was added. The results are set out in Table I:

TABLE I.—*Acidification of Direct-reaction Serum (Human).*

Serum (c.c.).	N/2 HCl (c.c.).	Water (c.c.).	Reagent (c.c.).	Final pH of mixture.	Colour.
0·5	0·1	0·4	0·5	1·68	Immediate scarlet.
0·5	0·2	0·3	0·5	1·44	„ violet-red.
0·5	0·3	0·2	0·5	1·24	Prompt violet-red.
0·5	0·4	0·1	0·5	1·12	Very delayed violet-red.
0·5	0·5	0·0	0·5	1·07	„ „

It will be seen that an immediate reaction occurred at a pH as low as 1·24, below which value the appearance of the colour was much delayed. With decreasing pH the colour tended to become more violet.

The experiment was repeated, using serum from a rabbit with obstructive jaundice (Table II). Similar results were obtained as in the foregoing experiment.

TABLE II.—*Acidification of Direct-reaction Serum (Rabbit).*

Serum (c.c.).	N/2 HCl (c.c.).	Water (c.c.).	Reagent (c.c.).	Final pH of mixture.	Colour.
0·5	0·0	1·0	0·5	1·59	Immediate rose.
0·5	0·1	0·9	0·5	1·40	,, ,,
0·5	0·2	0·8	0·5	1·25	,, ,, paler.
0·5	0·3	0·7	0·5	1·16	,, ,, ,,
0·5	0·4	0·6	0·5	1·09	Delayed purple.
0·5	0·5	0·5	0·5	1·03	,, ,,
0·5	1·0	0·0	0·5	0·84	No colour.

With a view to ascertaining whether the "direct-reaction" pigment prepared from bile has a similar pH range of reactivity to that of the pigment of obstructive jaundice, we repeated the above experiment upon a solution of the "direct-reaction" pigment in human serum. It was found (Table III) that the relationship between pH and reactivity was, in the main, identical with that seen in the case of icteric serum. The colours seen with the artificial icteric serum made from "direct-reaction" pigment tended only to be a little more violet.

TABLE III.—*Acidification of a Solution of "Direct-reaction" Pigment in Serum.*

Serum (c.c.).	N/2 HCl (c.c.).	Water (c.c.).	Reagent (c.c.).	Final pH of mixture.	Colour.
0·5	0·1	0·4	0·5	1·66	Immediate crimson.
0·5	0·2	0·3	0·5	1·43	Prompt violet-red.
0·5	0·3	0·2	0·5	1·27	,, ,,
0·5	0·4	0·1	0·5	1·10	Very delayed red-violet.
0·5	0·5	0·0	0·5	1·00	,, ,, violet.

These findings, which we have repeatedly confirmed, strengthen our belief in the identity of the pigment which we isolated from bile with the pigment of direct-reaction serum. As in the case of direct-reaction serum, marked adsorption of the pigment occurs on addition of alcohol to a solution of "direct-reaction" pigment in serum.

EFFECT OF ALKALINIZATION OF DIRECT-REACTION SERUM.

The serum of a rabbit, taken 48 hours after ligation of the common bile-duct, was treated with progressively increasing amounts of N/2 sodium hydroxide in a manner analogous to the foregoing experiments. It was found that no appreciable diazo-reaction occurred when the final pH of the reaction mixture was greater than 5·0.

EFFECT OF ALKALINIZATION OF INDIRECT-REACTION SERUM.

We have shown (Griffiths and Kaye, 1930) that pure bilirubin suspended in serum gives no prompt direct reaction until the final pH of the reaction mixture lies between 5·0 and 7·0. Similar conditions were necessary in the case of a serum from a patient with familial acholuric jaundice, which serum gave no direct, but a marked indirect reaction. On treatment with chloroform the pigment passed into the chloroform with great ease; there was no adsorption of the pigment by the protein precipitate on addition of alcohol to the serum. The effect of alkalinization of this serum upon the diazo-reaction will be seen from Table IV. This serum developed a prompt red reaction at the same pH level which we found to be necessary before reaction occurred in a serum-suspension of pure bilirubin.

TABLE IV.—*Alkalinization of Indirect-reaction Serum.*

Serum (c.c.).	N/2 NaOH (c.c.).	Water (c.c.).	Reagent (c.c.).	Final pH of mixture.	Colour.
0·5	0·00	0·05	0·5	2·44	No colour.
0·5	0·05	0·45	0·5	3·87	Immediate deep yellow.
0·5	0·10	0·40	0·5	5·54	,, scarlet.
0·5	0·15	0·35	0·5	8·52	Orange-yellow.
0·5	0·20	0·30	0·5	10·48	Greenish orange.
0·5	0·25	0·25	0·5	11·02	No colour; oxidized.

DISCUSSION.

We particularly wish to call attention to the similarity between a solution of "direct-reaction" pigment in serum and direct-reaction icteric serum, as shown by the very comparable conditions under which they react with diazo-reagent. This fact, together with other properties which they have in common, seems to leave no doubt that the pigment in direct-reaction blood-serum is identical with the "direct-reaction" pigment of bile. This pigment is quite distinct from that giving the indirect reaction, namely bilirubin, which reacts with diazo-reagent at quite a different pH level, and has different solubility properties, etc.

It was observed that the diazo-reaction given by the rabbit sera was always red, with a variable tendency to assume a violet tint subsequently. This type of reaction might be called "biphasic-direct" by some workers. Like Roberts (1928), however, we have not encountered the classic "immediate violet" reaction, although human sera and solutions of "direct-reaction" pigment in serum tended to take on a violet tint more rapidly than was the case with rabbit sera. It may be that further work with different technique from that employed by us (as, for example, that cited by Beaumont and Dodds, 1926) would give somewhat differently coloured reactions. We have noticed that variations in the concentration of pigment, and in the final pH of the reaction mixture, have a bearing upon the degree of violet colour in the complete reaction.

We have confirmed the observations of Davies and Dodds (1927), that the

buffering power of normal human serum and of human direct-reaction serum is the same; we find that this applies also to human indirect-reaction serum. These findings do not support the hypothesis of M'Gowan (1930) that the nature of the diazo-reaction is dependent upon the buffer-capacity of the serum.

SUMMARY.

1. In a series of six rabbits, ligation of the common bile-duct was followed by early and definite icterus, the serum giving a prompt direct van den Bergh reaction.

2. Direct-reaction human serum, and the serum of rabbits after bile-duct ligation, resemble in their properties a solution in serum of the "direct-reaction" pigment which we have shown to be present in bile.

3. Human indirect-reaction serum resembles in its properties a suspension in serum of pure bilirubin.

4. We have shown that the "direct-reaction" pigment yields a diazo-reaction over a wide range of pH, viz. when the pH of the mixture after addition of reagent lies between 1·2 and 5·0 approximately; on the other hand, the pigment of indirect-reaction serum, like bilirubin, yields no prompt direct reaction until the final pH of the reaction mixture is adjusted to about 6·0 by addition of alkali to the serum before mixing with diazo-reagent.

Our thanks are due to Prof. Hugh MacLean for his help and encouragement in this work and for facilities enjoyed, and to Dr. H. K. Goadby, who kindly examined the livers of the experimental animals and furnished the microscopical findings. One of us (W.J.G.) is indebted to the Medical Research Council for a grant.

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NORMAL FLOCCULATING H- AND GRANULATING O-AGGLUTININS FOR *BACILLUS TYPHOSUS* IN SERA OF DIFFERENT SPECIES.

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Forwarded for publication October 16th, 1930.

THE investigations of Weil and Felix have demonstrated beyond question that two types of agglutinogens can occur in a bacterial suspension: labilotropic H-receptors and stabilotropic O-receptors. Analogous to this, sera can also contain two types of agglutinins: coarsely flocculating H-agglutinins and finely granulating O-agglutinins. It is now generally accepted that in typhoid usually both H- and O-agglutinins increase in the serum in the course of the disease. As regards the nature of normal agglutinins for *B. typhosus*, however, the data contained in the current literature regarding the presence of normal coarsely flocculating agglutinins in sera are contradictory. Schiff (1922) could not ascertain the presence of large flaking agglutinins for several bacilli of the enteric- and proteus-group in sera of different species. He concludes, therefore, that all normal agglutinins belong to the small flaking, stabilotropic type of Weil and Felix. This conclusion corresponds with the results of Rotky (1921), who could not demonstrate labilotropic agglutinins for typhoid, Gärtner and fowl typhoid in normal human sera. Pijper (1930) also states in a recently published paper that normal agglutinins are of the O-type only.

In contra-distinction to this, Breinl (1920) in some cases showed the presence of large flaking agglutinins for typhoid, paratyphoid A and B and Gärtner in normal bovine sera. This was confirmed by Olitzki (1928), also in bovine sera, and by Felix and Olitzki (1929) in horse-sera. Smith, McVie and Newbold (1930) demonstrated the presence of labilotropic typhoid agglutinins in 14·9% of 302 human sera. In one case the titre amounted to as much as 1 : 5120.

The contradictory results of the various investigators are probably caused by dissimilar technique. Rotky experiments with "typhoid"—evidently a living suspension of *B. typhosus*—and with the alcohol-treated typhoid suspension of Bien and Sonntag. In this suspension the alcohol renders the large flaking receptors inactive. His conclusion is based on the fact that the titres of both series of agglutinations are completely identical. He does not state particulars as to the appearance of the flakes, nor as to the rapidity of the agglutination. Absorption experiments were not carried out. It is certainly not allowable to form the conclusion that H-agglutinins are absent on the grounds of these scarce data.

In several normal sera of man, sheep and cow, Schiff observed that the agglutination commenced after 15 minutes. The floccules were all of the coarse type. Thus his remark, later in the same paper, that all agglutinations were of the finely granulated type is incorrect. However, absorption with heated antigen removed all agglutinins from the serum. From this he concludes that all normal agglutinins belong to the stabilotropic type. Rapidly appearing, large and loose clumps are characteristic for labilotropic H-agglutination, however. It seems to be indicated that the manner in which he removed all O-agglutinins from the serum was so radical that the H-agglutinins were also destroyed. This point will be discussed later. Smith, McVie and Newbold, on the contrary, frequently demonstrated large flaking H-agglutination of *B. typhosus*. They investigated human sera, received for the Wassermann test. Nothing was known of the history of the patients. Therefore it is not certain that they had not suffered from typhoid fever previously, nor that they had not been vaccinated against typhoid. Both factors favour the H-agglutinability, thus rendering the percentage of positive findings too high.

The following experiments were undertaken with the view of investigating in detail the presence of H-agglutinins in normal sera of different species: guinea-pig, rabbit, horse, ruminants and man.

TECHNIQUE.

1. *Agglutination.*

The H-antigen used was prepared from a formolized broth culture (Dreyer, 1909) of a strain of *B. typhosus*, No. 18, isolated in this laboratory. The strain was morphologically smooth, and at no period of incubation showed any signs of sedimentation. The O-antigen consisted of an alcohol-treated suspension of the same strain, prepared according to Bien and Sonntag (1917). For all agglutinations receptors of the same batch were used. The agglutination was read with the agglutinoscope of Kuhn and Woithe after 2 hours' incubation at 37° and then, since small flaked agglutination appears rather slowly, after 18 hours at room temperature (*circa* 22° C.).

2. *Absorption.*

In connection with the remarks about Schiff's technique, two preliminary experiments were carried out. Schiff absorbs 5 c.c., 20-fold diluted serum, during 2 hours at 37° in the growth of one or two 24-hour agar Petri dishes. When two dishes are used, absorption with the bacilli of the first plate is carried out first. After centrifuging, the supernatant fluid is mixed with the bacilli of the second dish for another 2 hours. Moreover, Schiff agglutinates and absorbs with a bacterial suspension boiled for 30 minutes. In the first place, I have investigated the influence of different quantities of boiled bacilli (Table I).

Table I shows that absorption with the growth of $\frac{1}{4}$ plate is sufficient to remove all stabilotropic O-agglutinins from the serum. The labilotropic H-agglutinins are not injured. If absorption is carried out with the growth of $\frac{1}{2}$ or 1 dish, however, almost all the labilotropic H-agglutinins are rendered

inactive. The table also shows that the boiled growth of 1 dish does not remove the "alcohol" arglutinins.

TABLE I.—*Absorption with Boiled Antigen. Serum Buffalo 9; Ty. 18.*

		Reading after 2 hours' incubation + 18 hours' room temperature.		
		Formolized suspension.	Alcohol-treated suspension.	Boiled suspension.
Normal serum	1:25	++	+	+
	1:50	++	+	+
	1:100	+	+	+
	1:150	±?	−	+
	Control	−	−	−
After absorption with the growth of $\frac{1}{8}$ dish	1:25	++	+	+
	1:50	++	+	+
	1:100	+	+	+
	1:150	−	−	±
	Control	−	−	−
After absorption with the growth of $\frac{1}{4}$ dish	1:25	++	+	−
	1:50	++	+	−
	1:100	+	+	−
	1:150	−	−	−
	Control	−	−	−
After absorption with the growth of $\frac{1}{2}$ dish	1:25	+	+	−
	1:50	±	+	−
	1:100	−	±	−
	1:150	−	−	−
	Control	−	−	−
After absorption with the growth of 1 dish	1:25	−	+	−
	1:50	−	±	−
	1:100	−	±	−
	1:150	−	−	−
	Control	−	−	−

++ = strong agglutination with partial sedimentation. + = distinct agglutination without sedimentation. ± = trace of agglutination.

In the next experiment absorption is carried out with an alcohol-treated suspension of *B. typhosus* (Table II).

Here also absorption with $\frac{1}{4}$ dish is sufficient to remove all O-agglutinins, whereas the H-agglutinins are not injured. After absorption with the growth of $\frac{1}{2}$ or 1 dish, however, some of the H-agglutinins are rendered inactive. Finally, it shows that, after absorption with alcohol-treated suspension, the "boiled" O-agglutinins are also removed, contrary to what had been ascertained in the first experiment regarding the "alcohol" O-agglutinins after absorption with boiled antigen. From this it follows that both suspensions (boiled and alcoholic) are not identical. Gardner (1928) has already pointed to this fact.

It appears that the boiled suspension contains less O-antigen than the alcohol-treated suspension. Therefore in all experiments I have used an alcoholic suspension. Both experiments show that the growth of $\frac{1}{4}$ dish is sufficient to remove all O-agglutinins from 5 c.c. of a 20-fold dilution of serum in 2 hours at 37° C. The H-agglutinins are not injured. Therefore, contrary to Schiff, I have always carried out the absorption with the growth of $\frac{1}{4}$ plate. This quantity has proved to be sufficient in all instances.

TABLE II.—*Serum Buffalo 9; Ty. 18. Absorption with Alcohol-Antigen.*

		Formolized solution.	Alcohol-treated suspension.	Boiled suspension.
After absorption with the growth of $\frac{1}{8}$ dish	1:25	++	+	+
	1:50	++	+	+
	1:100	+	−	−
	1:150	−	−	−
	Control	−	−	−
After absorption with the growth of $\frac{1}{4}$ dish	1:25	++	−	−
	1:50	++	−	−
	1:100	+	−	−
	1:150	−	−	−
	Control	−	−	−
After absorption with the growth of $\frac{1}{2}$ dish	1:25	+	−	−
	1:50	+	−	−
	1:100	−	−	−
	1:150	−	−	−
	Control	−	−	−
After absorption with the growth of 1 dish	1:25	±	−	−
	1:50	−	−	−
	1:100	−	−	−
	1:150	−	−	−
	Control	−	−	−

RESULTS.

1. *Guinea-pigs.*

TABLE III.

Total.	O-agglutinins.		H-agglutinins.	
	Negative.	Positive, 1:25.	Negative.	Positive.
20	18	2	20	0

H-agglutinins could never be demonstrated. Only 2 animals out of 20 showed a small amount of O-agglutinins (titre 1:25).

2. *Rabbits.*

TABLE IV.

Total.	O-agglutinins.				H-agglutinins after absorption.	
	Negative.	Positive.			Negative.	Positive.
		1:25.	1:50.	1:100.		1:100.
24	6	13	4	1	23	1

Out of 24 rabbits, only 6 proved completely free from O-agglutinins. Of the remaining 18, only 1 had a titre of 1 : 100; the titre of all others was lower. One rabbit showed H-agglutinins (1 : 100); its O-titre amounted to 1 : 25 only.

3. *Horses.*

TABLE V.

Total.	O-agglutinins.					H-agglutinins after absorption.		
	Negative.	Positive.				Negative.	Positive.	
		1:25.	1:50.	1:100.	1:200.		1:25.	1:50.
14	0	2	8	3	1	9	2	3

All horses showed O-agglutinins, 5 also H-agglutinins. The relations between H- and O-titre are shown in Table VI.

TABLE VI.

Horse.	H-titre.	O-titre.
5	1:50	1:200
36	1:50	1:50
37	1:25	1:50
39	1:25	1:100
40	1:50	1:100

This table shows that the O-titre was usually higher than the H-titre. Thus the average O-titre was also higher than the average H-titre (100 : 40).

4. *Sheep and Goats.*

TABLE VII.

Total.	O-agglutinins.				H-agglutinins after absorption.			
	Negative.	Positive.			Negative.	Positive.		
		1:25.	1:50.	1:100.		1:25.	1:100.	1:200.
2 goats	0	2	...	....	0	1	1	...
2 sheep	0	...	1	1	0	...	...	2

All 4 animals possessed both H- and O-agglutinins.

5. *Buffaloes* (Bos bubalus L.).

TABLE VIII.

Total.	O-agglutinins.				H-agglutinins after absorption.			
	Negative.	Positive.			Negative.	Positive.		
		1:25.	1:50.	1:100.		1:25.	1:100.	1:200.	1:400.
12	2	3	2	5	0	2	5	2	3

All buffaloes showed H-agglutinins and 10 O-agglutinins. In two cases H- and O-titres were equal. In all the remaining cases the H-titres were higher. Therefore the average H-titre (180) is higher than the average O-titre (56).

6. *Men.*

TABLE IX.

Total.	O-agglutinins.				H-agglutinins after absorption.				
	Negative.	Positive.			Negative.	Positive.			
		1:25.	1:50.	1:100.		1:25.	1:50.	1:100	1:400.
16	7	3	5	1	9	1	1	4	1

The series is rather small, because of the difficulty to discover men here who have never suffered from typhoid and have never been vaccinated against typhoid either. Five men did not possess any agglutinins, 4 only O-, 2 only H-, and 5 both types. The average H-titre is higher than the average O-titre.

DISCUSSION.

The experiments show that normal H-agglutinins for *B. typhosus* are not infrequently present in normal sera of several species of animals and of men. H-agglutinins often occur in a rather high dilution; 1 : 400 was the highest titre ascertained in man. The experiments also show that the conditions are not similar in all species of animals. H-agglutinins could never be demonstrated in 20 guinea-pigs; in 24 rabbits only once, in 14 horses 5 times, in 16 men 7 times, in 16 ruminants (2 sheep, 2 goats, 12 buffaloes) in all instances. O-agglutinins occurred twice in 20 guinea-pigs, 18 times in 24 rabbits, 14 times in 14 horses, 14 times in 16 ruminants and 7 times in 16 men. Therefore as regards both types of agglutinins, men occupy an intermediate position.

If we consider the presence of agglutinins generally, without taking into consideration the differences between H- and O-agglutinins, it shows that guinea-pigs possess the least agglutinins (90% negative); then follow rabbits and men (25% negative); finally horses and ruminants (0% negative). If considered in this way, man also occupies the same intermediate position. This corresponds with Bürgi's experiments (1907), who devised the following series: cow, horse, goat, sheep, rabbit, man, guinea-pig, in which cows possess the largest, guinea-pigs the smallest amount of agglutinins for several

micro-organisms. In a recent paper Gibson (1930) arrived at the same conclusion as regards normal agglutinins for many microbes.

The question as to whether any connection can be established between the capacity of producing normal O- and H-agglutinins must also be considered. So far as the small numbers allow a conclusion, it appears that in men and ruminants, normal H-agglutinins are more easily produced than O-agglutinins. The average titres bear a proportion of 2·5 : 1 and 2·7 : 1 respectively. In horses, on the contrary, the proportion is just reversed (1 : 3).

In order to investigate the distribution of the normal H- and O-agglutinins in the different proteins of the serum, I have fractionated a few normal sera.

Nearly all investigators agree that albumen is free or almost free from immune properties, independent of the method in which the fractionation has been carried out.

As regards the distribution of antibodies in euglobulin and pseudoglobulin, however, the views are divergent. Next to the opinion of Kapsenberg and Rispens (1927), and of Laubenheimer (1928), that agglutinins for typhoid and paratyphoid are equally divided in pseudoglobulin and euglobulin, different theories regarding agglutinins and other antibodies have been advanced. Laubenheimer and Vollmar (1926) demonstrated the greater part in euglobulin after separation of the serum with ammonium sulphate. If the fractionation has been carried out according to Bechhold's electro-ultra-filtration process, all hæmolysins are contained in the euglobulin fraction. These results are in contradiction to those of Ruppel *et al.* (1923), who fractionated electro-osmotically. They found hæmolysins, diphtheria and tetanus antitoxin in pseudoglobulin, agglutinins for *B. erysipelatis suis* for the greatest part in euglobulin. Doerr and Hallauer (1927) state that precipitins and anaphylactic antibodies are completely contained in euglobulin after electro-ultra-filtration of the serum. Otto and Sukiennikowa (1924) used the electro-osmotic method: hæmolysins were contained principally in the euglobulin fraction. Locke and Hirsch (1924) found hæmolysins after electro-dialyzation in the pseudoglobulin exclusively. Pick demonstrated the presence of typhoid agglutinins in euglobulin of goats, rabbits and guinea-pigs, in pseudoglobulin of horses, however.

The diverging results of the experimenters are certainly partly caused by the fact that the fractionation has been carried out by different methods.

It is now established beyond question that the fractions which we call "euglobulin" and "pseudoglobulin" differ according to the process in which we have obtained them—precipitation with ammonium sulphate, electro-ultra-filtration or electro-osmosis.

All quoted experiments have been carried out with immune sera, usually of a very high titre. Landsteiner and Calvo (1902) have fractionated normal horse-sera with ammonium sulphate and have examined the agglutinative power of the fractions—in the first place for red blood-cells of rabbit, man, guinea-pig and pigeon. The euglobulin contained most agglutinins, the pseudoglobulin much less. In the second place, agglutination of cholera and typhoid bacilli was examined. Here, also, agglutinins were contained in euglobulin for the greater part. The differences with pseudoglobulin were not so striking, however, as was the case with normal hæmagglutinins. Gibson

(1930) found the normal agglutinins to be present for the greater part in the carbonic acid insoluble fraction of the serum, which contains the euglobulin and part of the pseudoglobulin.

The only experimenter who has paid attention to the different types of agglutination is Beck (1926). He used sera of rabbits immunized against different micro-organisms of the Samonella group. These sera were separated electro-osmotically. He ascertained that the small flaking group agglutinins were contained in the pseudoglobulin-albumen fraction only, the specific agglutinins (large and small flaking) almost exclusively in the euglobulin.

Mr. C. A. Kramers has kindly fractionated 2 normal buffalo sera and 1 normal cow serum. Ruppel's electro-osmotic method was used. Tables X, XI and XII show the results.

TABLE X.

Buffalo 1.	H-agglutinins.	O-agglutinins.
Serum	1:200	1:100
Albumen	0*	0*
Pseudoglobulin	0*	0*
Euglobulin (corrected)†	1:200	1:75

* 0 in this and subsequent tables means negative in a dilution 1:5.
† "Corrected" means that this titre is yielded by the euglobulin, resolved in physiological salt solution to a total volume equal to the original amount of serum.

Table X shows that albumen and pseudoglobulin are free from both types of agglutinins, which are contained in the euglobulin exclusively. The O-titre of the euglobulin is lower than of the serum. This is probably caused by the loss of euglobulin during the process of fractionation (16·5% of the total globulin was lost). Evidently the determined O-titre of the serum (1:100) was so near the limit that the loss of euglobulin was sufficient to reduce this titre to 1:75. The H-titre was probably so much higher than 1:200 that, even after the loss, the same titre could still be attained.

TABLE XI.

Buffalo 2.	H-agglutinins.	O-agglutinins.
Serum	1:100	1:100
Albumen	0	0
Pseudoglobulin	0	0
Euglobulin (corrected)	1:100	1:100

Table XI also shows that all agglutinins are contained in the euglobulin fraction of the serum. The titre was not influenced by the loss of globulin. The same was found in horse-serum.

TABLE XII.

Horse 1.	H-agglutinins.	O-agglutinins.
Serum	1:25	1:100
Albumen	0	0
Pseudoglobulin	0	0
Euglobulin (corrected)	1:25	1:100

In all three instances in Table XII both albumen and pseudoglobulin proved free from agglutinins. The opinion of Pick, that in horses the immune agglutinins were contained in pseudoglobulin, could not be confirmed for normal agglutinins. Both types of agglutinins (O and H) are contained quantitatively in the euglobulin fraction.

CONCLUSIONS.

1. Normal agglutinins for *B. typhosus* in sera of man, guinea-pig, rabbit, horse, sheep, goat and buffalo can be of the O- and of the H-type.

2. It appears that in men and ruminants normal H-agglutinins occur more frequently than normal O-agglutinins, contrary to what has been observed in horses and rabbits.

3. Sera of guinea-pigs show the lowest content of normal typhoid agglutinins. In the ascending scale sera of rabbits and men follow, reaching to horses and ruminants at the apex.

4. After electro-osmotic fractionation of the serum, both types of normal agglutinins for *B. typhosus* are found in the euglobulin fraction exclusively.

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THE RESPONSE OF GRAIN-FED PIGEONS TO SUBSTANCES EFFECTIVE IN PERNICIOUS ANÆMIA.

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Received for publication October 1st, 1930.

Analysis of the recent literature concerned with the treatment of human anæmias shows that those anæmias, which respond in a dramatic way to liver therapy, are characterized by an increase in size of the circulating red blood-cells associated with "megaloblastic" hyperplasia of the bone-marrow (Vaughan [1930*a*]). Included in this group are Addisonian pernicious anæmia, at least certain cases of tropical sprue, certain cases of anæmia associated with pregnancy, often called the "pernicious anæmia of pregnancy," and anæmias associated with total gastrectomy and intestinal obstruction presenting the same blood-picture as pernicious anæmia.

Peabody (1927), elaborating the observations of earlier workers on the bone-marrow of pernicious anæmia, demonstrated that during remissions produced by liver therapy the megaloblastic hyperplasia characteristic of this disease gave place to an apparently normal bone-marrow. Reports on the bone-marrow of sprue and the so-called "pernicious anæmia of pregnancy" are rare. Mackie and Fairly (1929) have recently shown that in many cases of sprue it is similar to that in Addisonian pernicious anæmia, while in the ill-defined condition, "pernicious anæmia of pregnancy," there is some evidence to suggest that a similar megaloblastic hyperplasia is also present (Vaughan, 1930*a*).

The group of anæmias characterized by this megaloblastic hyperplasia must be regarded as due to the deficiency of some substance or substances essential for the normal function of the bone-marrow. Minot and Murphy (1926) showed that liver and later that kidney (1927) fed in sufficient quantity was an unfailing remedy in pernicious anæmia. Castle and Locke (1928–1929) published observations which demonstrated that beef muscle digested with normal gastric juice was equally effective. Sturgis and Isaacs (1929) have more recently shown that hog's stomach, if suitably prepared, is also adequate to relieve the anæmia.

The bone-marrow of the healthy grain-fed pigeon is likewise characterized by the predominance of megaloblastic tissue. It has been shown that this bone-marrow is peculiarly susceptible to the effect of liver-feeding (Barlow, 1930; Muller, 1927, 1929). It therefore appeared possible that the administration to healthy grain-fed pigeons of substances capable of

relieving conditions in man, associated with a megaloblastic bone-marrow, might produce a response in the bird similar to that found in clinical cases.

In such human cases the administration of liver or its substitutes is followed by a temporary increase in the circulating reticulocytes, which rise to a maximum level, dependent, as Minot, Cohn, Murphy and Lawson (1928*b*) have shown, upon the original level of the red blood-cell count and upon the amount of effective material given. This response of the reticulocytes takes place over a period of about ten days. The height to which the reticulocytes rise, in any one case of pernicious anæmia, can be calculated from data given by Minot and his associates (1928*b*), and therefore the response of the reticulocytes obtained in pernicious anæmia can serve to estimate the adequacy of the material employed.

The present paper deals particularly, therefore, with the effect of the administration of liver preparations effective in pernicious anæmia and other test material on the reticulocyte count in grain-fed pigeons.

EXPERIMENTAL METHODS.

Healthy grain-fed pigeons were used which had shown a steady body-weight and a reticulocyte count not above 12% of the total red blood-cells during a short control period. Birds which had been fed upon a grain diet for some weeks were usually found to have about 10% of reticulocytes in the circulating blood. They were often present in somewhat greater numbers when the birds were first sent from the market. The significance of this change will be discussed later. After treatment was started the birds were weighed and the reticulocytes counted at approximately the same hour each day. In determining the percentage of reticulocytes a modification of the method described by Hawes (1909) and Cunningham (1920) was employed.*

Total red-cell counts and hæmogloblin determinations were made the day treatment was started and the day after the last dose of test material was given. In some cases additional determinations were made during the period of alteration in the reticulocyte count. The hæmoglobin concentration was read in a Duboscq colorimeter against the Newcomer standard. Intravenous injections were made into a vein of the leg, using a volume of 2 c.c.

A number of birds were killed at various intervals after the administration of a test material. Pieces of spleen, liver, kidney and bone-marrow from the femur and radius were taken for section and stained in the manner previously described (Muller, 1927).

The experiments were divided into five groups, and concern observations on the influence of—

* Considerable difficulty was found in obtaining satisfactory preparations in which no polychromasic cells occurred, owing to the large number of reticulocytes present in the rather thick blood of the healthy pigeon, in which the stain was also taken up by the nuclei of the red blood-cells. To ensure complete staining of the reticular material brilliant cresyl-blue films, somewhat thicker than those used in making preparations of human blood, were made on both of the cover-slips to be employed. Five hundred red cells at least were then counted on each cover-slip and the percentage of reticulocytes determined. Preparations in which polychromasic cells were present were discarded as likely to give too low a count.

1. Liver preparations* known to be effective in the treatment of pernicious anæmia.
2. Liver preparations which had proved ineffective in the treatment of pernicious anæmia, or in which an attempt had been made to destroy the active principle.
3. Liver preparations† which had received no clinical test.
4. A series of control substances.
5. Gastric juice and beef-steak.

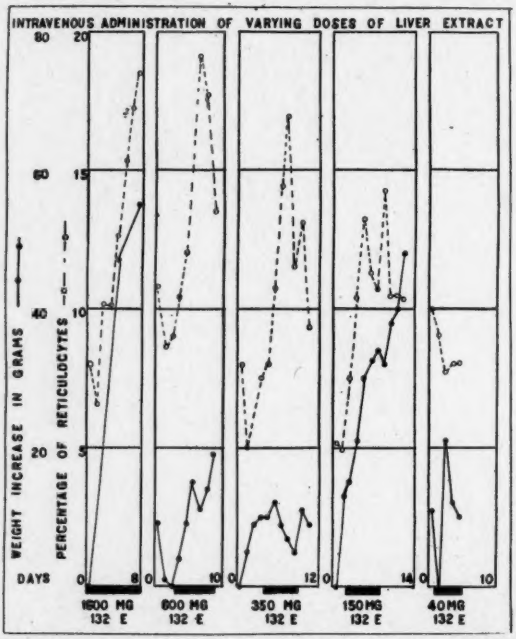
INTRAVENOUS ADMINISTRATION OF VARYING DOSES OF LIVER EXTRACT
WEIGHT INCREASE IN GRAMS
PERCENTAGE OF RETICULOCYTES
DAYS
1600 MG 132 E
800 MG 132 E
350 MG 132 E
150 MG 132 E
40 MG 132 E

FIG. 1.—The response of the reticulocytes and the weight of healthy grain-fed pigeons to the intravenous administration of varying doses of a liver preparation effective in pernicious anæmia.

Group I.—Preparations Effective in Pernicious Anæmia.

A group of five pigeons were given a relatively impure preparation, 124 E, by intravenous injection (Cohn, McMeekin and Minot, 1930) in doses varying from a total of 1600 mgm. to 40 mgm. A constant response of the reticulocytes was shown in the birds given the higher doses. There was a rise in the reticulocytes from an average control level of 9·2% to about 18% in the course of 4 to 8 days. This was accompanied by a rapid gain in the bird's weight.

* We are greatly indebted to Dr. Edwin J. Cohn and Dr. Thomas L. McMeekin, of the Department of Physical Chemistry of the Harvard Medical School, who made the liver preparations and supplied them for our experiments. The clinical tests were made on patients under the supervision of Dr. George R. Minot, who kindly supplied us with all the necessary information as regards the potency of the various preparations.

Fig. 1 shows that the reticulocyte response was of approximately the same order, whether the total dose, given in several injections over 6 days, was 1600 mgm. or 350 mgm. When a total dose of 150 mgm. was given in 25-mgm. doses each day for 6 days a definite response occurred but of smaller magnitude. A total dose of 40 mgm., however, yielded no response. A total dose of 150 mgm. given over a period of 6 days in equal doses, *i. e.* 25 mgm. daily, was adopted, therefore, somewhat arbitrarily, as a standard dosage in all subsequent experiments. Preparations which gave no response when given in this quantity and manner were considered inactive.

Fig. 2 shows the typical type of reticulocyte response that was obtained in birds receiving effective material. The reticulocytes rose from a level in the neighbourhood of 10%, at which level they had been during the control period, to one of about 18%. They then fluctuated at or about this latter level for 10 days before falling to normal.

Fig. 3 compares the reticulocyte response in a pigeon and in a patient with pernicious anæmia who were given the same liver preparation, at that time of unknown potency, at the same time. In both there was a satisfactory response. Discussion of the significance of variations in this response is reserved until all the experimental data has been given. The reticulocytes were not only increased in number after the administration of an effective preparation, but the majority were more immature in type than any seen in the circulating blood before treatment.

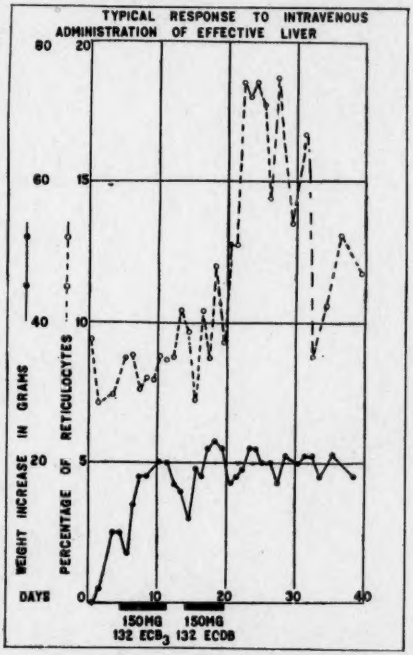

FIG. 2.—A typical reticulocyte response in a healthy grain-fed pigeon following a total dose of 150 mgm. of a liver preparation (132 ECDB), effective in pernicious anæmia, administered intravenously for six days in 25-mgm. doses on each of six successive days. The lack of response to a liver preparation (132 ECB3), ineffective in pernicious anæmia, is also shown.

Seventeen birds in all were given effective preparations by intravenous administration. Table I shows that the purer preparations were effective in doses as small as 20 mgm., given in two equal doses of 10 mgm. on two successive days. Only one potent preparation, 134 ZHA 2, failed to give a response in pigeons. It was apparently effective but obviously not highly potent, for in the one patient given the material it produced only a weak reticulocyte reaction. Owing to scarcity of material it was impossible to test this preparation on pigeons using the standard total dose of 150 mgm. Doses, therefore, of 50 and 60 mgm. only were employed, and since this was a relatively impure extract, the small quantity of effective material given probably

explains the negative result in the birds. Another but much purer preparation, 134 ZH2A, was also given to pigeons in an extremely small total dose of 20 mgm. The reticulocytes rose to a level of only 14·6%, but as the majority were of a definitely immature type this can be regarded as a positive result.

TABLE I.—*Response in Healthy Grain-fed Pigeons to Intravenous Administration of Liver Preparations Effective in Pernicious Anæmia.*

Pigeon number.	Preparation number.	Dose in mgm.	Percentage of reticulocytes. Average in control period.	Maximum response.	Body-weight gain in gm.
182	132 E	2400	10·0	17·0	38
181	132 E	2200	11·2	20·1	38
175	132 E	1600	8·0	18·5	55
174	132 E	1400	10·0	20·9	41
189	132 E	600	5·7	18·8	15
188	132 E	350	7·1	16·9	18
190	132 E	150	6·0	14·2	64
191	132 E	40	9·7	12·5	0
208	134 ECDB	150	9·3	18·7	0
213	134 ECDB	60	8·7	20·1	0
244	134 Z	20	10·9	15·9	0
228	134 ZHB	60	11·0	21·0	0
227	134 ZHB	60	9·7	22·1	0
220	134 ZHA2	50	9·8	13·8	0
206	134 ZHA2	60	11·0	11·0	0
242	134 ZH2A	20	11·2	14·6	0
240	135 ZHA	20	10·4	20·2	0

TABLE II.—*Response in Healthy Grain-fed Pigeons to the Oral Administration of Liver Extract No. 343 N.N.R. Effective in Pernicious Anæmia.*

Pigeon number.	Dose in grm.	Percentage of reticulocytes. Average in control period.	Maximum response.	Body-weight gain in gm.	Remarks.
190	20·0	9·6	17·4	16	...
187	11·0	8·0	16·8	22	...
197	6·0	11·2	17·7	0	...
211	6·0	10·1	16·6	4	Killed.
223	6·0	10·1	16·9	0	...
206	6·0	10·7	15·8	0	...
220	6·0	11·2	23·5	0	...
209	3·0	10·2	21·1	19	...
236	3·0	11·5	19·0	4	...
230	3·0	9·5	14·8	0	Sepsis.
230	6·0	9·5	19·6	0	Sepsis improved.
215	1·5	11·5	15·8	9	...

Eleven pigeons were given liver extract No. 343 N.N.R. by mouth. The response is shown in Table II. The results appear to be the same whether the total dose was 20 gm., or 1·5 gm. given in equally divided doses over a 6-day period. One bird only failed to show the typical reticulocyte response, and was found on the fifth day of treatment to have a severely ulcerated mouth.

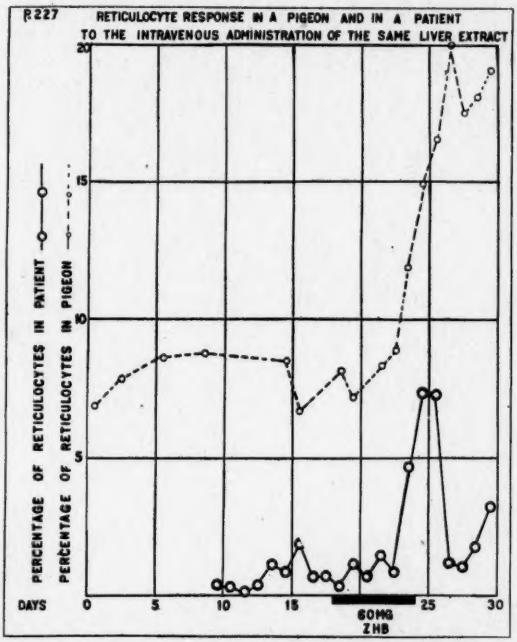

FIG. 3.—The reticulocyte response in a healthy grain-fed pigeon following a total dose of 60 mgm. of an effective liver preparation (ZHB) and the reticulocyte response in a patient with pernicious anæmia who received 1·6 gm. of the same liver preparation, beginning treatment on the same day. The patient had previously had a reticulocyte response to another effective preparation, and therefore the second, as is often the case, was not quite typical. Note how in both the reticulocytes started to rise on the sixth day.

Sepsis is known to interfere with the action of the effective principle in pernicious anæmia, and the observations on the pigeons just mentioned suggest it plays the same *rôle* in pigeons. When the dose was increased from a total of 3 gm. to 6 gm., coincident with an improvement in the condition of the mouth there was a satisfactory rise in the reticulocytes.

Group II.—Preparations which had Proved Ineffective in the Treatment of Pernicious Anæmia.

Two preparations, each of which had proved ineffective in treating single cases of typical pernicious anæmia, were tested on pigeons. The response to the first was completely negative, though the dose was increased to a total of

300 mgm. The bird later reacted to a known positive fraction. The second was apparently a pure amino-acid leucine. In some preliminary experiments both this preparation and commercial leucine appeared to give a similar reticulocyte response in pigeons. The experiments were, however, not well controlled and the point is being further investigated.

Riddle and Curtis (1929) have shown that it is possible to render liver extract No. 343 N.N.R. ineffective for pernicious anæmia by subjecting it to a pressure of 15 lb. and a temperature of 120° F. for 2 hours. A total dose of 6 gm. of extract treated in this manner was given to two pigeons and evoked no response. One bird later reacted well to a similar dose of potent liver extract, 343 N.N.R., which had not been autoclaved, while the second bird gave a typical response to another potent material.

TABLE III.—*Response in Healthy Grain-fed Pigeons to the Intravenous Administration of Liver Preparations which had not been Tested on Pernicious Anæmia Cases.*

Pigeon number.	Preparation number.	Dose in mgm.	Percentage of reticulocytes. Average in control period.	Maximum response.	Body-weight gain in gm.
197	132 EA2	150	9·6	14·5	0
202	132 EA2	60	9·9	13·4	0
204	132 EB2	150	10·5	23·7	28
203	132 EB2	150	9·9	18·8	10
196	132 EB2	150	9·5	17·5	0
191	132 EB2	125	9·7	19·8	21
206	132 EB2	60	9·2	20·0	0
212	132 ECB1	150	10·0	17·0	0
222	132 ECB1	150	6·1	15·9	0
210	132 ECB1	60	10·0	17·3	0
216	132 ECB1	60	9·5	37·0	0
221	132 ECB2	127·5	9·4	25·9	0
223	132 ECB2	50	11·3	12·4	0
218	132 ECB2	60	10·1	12·7	0
208	132 ECB3	150	9·1	10·4	0
197	132 ECB4	150	10·0	13·4	0
196	132 ECB4	150	9·3	10·3	0

Group III.—Liver Preparations which had not been Tested Clinically on Pernicious Anæmia.

Table III shows the results in the third group of birds which were given a preparation which had not been tested clinically. A preparation, 132 E, which gave a positive result in both patients and pigeons was divided by chemical methods by Dr. Cohn and his associates into two fractions, 132 EA1 and 132 EB1. Preparation 132 EA1 was active, as shown by a clinical test. Preparation 132 EB1 was not so tested. Two fractions prepared in exactly

the same way, but both untested clinically, were given to pigeons, 132 EA2 and 132 EB2. Preparation 132 EA2, as reference to Table III will show, was slightly active. Preparation 132 EB2 was also effective in producing a response in pigeons similar in every respect to those given by other fractions. Three birds reacted to a total dose of 150 mgm. and two birds to a dose of 125 and 60 mgm. Preparation 132 EB2 was then split by further chemical procedure into four parts, ECB1, 2, 3 and 4. Two of these, ECB3 and ECB4, were completely negative in pigeons. Preparation ECB1 gave a good response in as low a dose as 60 mgm. in two birds. ECB2 was effective in a large dose in one bird, but gave a negative response with reduced doses of 50 mgm. and 60 mgm. in two birds. It is clear, therefore, that the response obtained to these two preparations is due to some relatively specific factor, since it is present in considerable quantity in only one of the four fractions of an active preparation.

Group IV.—Control Substances.

1. *Sodium chloride.*—Three pigeons were given six daily injections of 2 c.c. of normal saline in order to determine that slight alteration in blood volume was not influencing the bone-marrow. In one the reticulocytes showed a very slight irregular fluctuation; in the other two birds there was no alteration in the count.

2. *Histamine.*—It was suggested that since certain impure preparations potent in pernicious anæmia are rich in histamine, this substance might affect the pigeons' bone-marrow. A pigeon was therefore given a total dose of 0·18 mgm. of histamine in equally divided doses of 0·03 mgm. for six days. The dose was then raised to a total of 0·36 mgm., which exceeds by far the amount which could possibly have been present in any of the materials tested on pigeons. There was no appreciable alteration in the reticulocyte count.

3. *Vitamin B.*—The less pure preparations, as already mentioned, caused a pronounced gain in the birds' weight. Since vitamin B is known to influence growth and appetite, and to be present in considerable quantity in whole liver and in some commercial extracts effective in pernicious anæmia, it was thought that the body-weight gain and possibly the reticulocyte rise in pigeons might be due to vitamin B. Two birds were therefore given a total dose of 1·3 c.c. of tici tici, which contains, relatively concentrated, the antineuritic factor of vitamin B—*i. e.* vitamin B 1 (G). This was given by intravenous injection in daily doses of 0·25 c.c. diluted to a volume of 2 c.c. in normal saline for a period of six days. There was no response, though the bird subsequently reacted to material effective in pernicious anæmia. Vitamin B 2 (F), or the pellagra-preventive factor, was fed in the form of a concentrate of yeast, which had previously been autoclaved to destroy vitamin B 1. The results were negative.

Group V.—Gastric Juice and Beef-steak.

Castle and his associates (1928–29, 1929*a*, 1929*b*) have shown that beef-steak previously digested with normal gastric juice is effective in inducing a remission in pernicious anæmia. Human gastric-juice when fed to pigeons

caused no reticulocyte response, but beef-steak fed to four birds gave a reticulocyte rise in every way similar to that obtained with effective liver preparations.

Danysz-Michel (1922) and Koskowski (1921–22) have shown that the gastric juice of pigeons contains hydrochloric acid and pepsin. Hammarsten (1908) demonstrated that hen's gastric juice contains small quantities of rennin. Though it has been shown that none of these substances is actually responsible for the specific reaction between gastric juice and beef-steak, it appears that avian gastric juice is not unlike that of man. It is reasonable, then, to assume that the pigeon's stomach is as capable as the human stomach of elaborating from beef muscle a substance effective in promoting blood production.

Casein proved ineffective as a substitute for beef muscle in both human cases of pernicious anæmia (Castle *et al.*, 1929*b*) and in pigeons.

ANALYSIS OF THE RETICULOCYTE RESPONSE.

As already mentioned, the character of the reticulocyte response in uncomplicated cases of pernicious anæmia following the administration of liver or its substitutes has been shown by Minot and his associates (1928*b*) to be dependent particularly upon two factors—(1) the amount of effective material administered; (2) the initial level of the red-cell count. It is probable that a third factor, namely, the precise state of the bone-marrow, is also of considerable importance, but for obvious reasons this is difficult to determine. If the first two factors are known it is possible, however, to calculate the height to which the reticulocyte count will rise in any case under treatment.

An analysis of the character of the reticulocyte response described as occurring when similar material is fed to pigeons is given in Table IV. It would appear that no such exact mathematical relationship exists between dosage and reticulocyte response in birds. The group receiving large doses of extract 132 E, it is true, show a difference of only 2·4% at the peak of the reticulocyte rise, but there is considerable variation in the eleven birds who were given liver extract No 343 N.N.R. by mouth irrespective of dosage, as reference to Table II shows. Differences in the amount absorbed from the gastro-intestinal tract may be in part responsible for such variability. There appear to be considerable variations in the duration of the response (3 to 10 days), and again in the day on which the reticulocytes start to rise (between the third and eighth day after treatment was begun). Burn (1930), however, has recently pointed out that all methods of biological essay depending upon animal tests are liable to such apparent variations unless certain mathematical considerations are taken into account and a large series of animals examined. Hitherto, birds from the market have been used whose age and previous conditions of life are unknown. It is clear that in future more completely standardized pigeons must be available if comparable results are to be obtained.

TOTAL RED-CELL COUNTS.

Red-cell counts were made on all birds before treatment and the day after the administration of the last dose of test material.

From an analysis of the total red-blood counts it appears justifiable to

TABLE IV.—*Character of the Reticulocyte Response in Healthy Grain-fed Pigeons given Liver Preparations Effective in Pernicious Anæmia.*

Number of pigeons.	Preparation number.	Total dosage given within seven days.	Reticulocyte percentage at peak of their rise.					Remarks.
			Maximum.	Minimum.	Average.	Day of initial rise.	Duration.	
3	132 E	2200–600 mgm.	20·9	18·5	19·5	4	6	Two killed 5th and 6th day.
2	132 E	350–150 mgm.	16·9	14·2	15·5	3	4	...
1	134 ECDB	150 mgm.	18·7	...	...	...	11	...
1	134 ECDB	60 „	17·9	...	...	7	...	Killed 11th day.
1	134 Z	20 „	15·9	...	...	5	3	...
2	134 ZHB	60 „	22·1	21·0	21·2	5–6	...	Killed 6th day.
1	134 ZH2A	60 „	14·6	...	...	3	4	...
1	135 ZHA	20 „	20·2	...	...	3	3	...
10	343 NNR	20–1·5 gm.	22·1	15·8	17·28	3–8	4–10*	...

* One bird only maintained a raised reticulocyte count for 27 days.

conclude that the administration of effective liver preparations is without any appreciable influence on the total number of circulating erythrocytes. Further, three birds who were fed upon liver extract 343 N.N.R. daily for long periods, varying from 4 to 10 weeks, had practically constant red blood-cell counts throughout this period when the reticulocytes were maintained at a high level in the r eighbourhood of 18%. A fourth bird maintained a similar high count when the dosage was increased. One bird only given preparation 134 E by intravenous injection for 22 days did not show any prolonged rise in the reticulocytes, though treatment was continuous.

Many investigators have studied the effect of forced liver-feeding on the total red-cell count in normal individuals. Berglund, Watkins and Johnson (1928) describe an irregular increase in total red blood-cells, but Cornell (1928), Minot *et al.* (1928*a*) and Neidhardt and Bannasch (1929) and others give negative reports. On the other hand, Minot and Murphy (1927) quote figures which suggest that it may be possible to force the total red-cell count in certain cases of pernicious anæmia at least as high as 6,300,000. It is possible that liver is able to exert a greater influence on the pathological bone-marrow present in this disease than upon the normal. It would also appear impossible to force the red blood-cell count of the pigeon above a certain level; the age rather than the number of circulating red blood-cells of the pigeon is affected by liver or its substitutes. McGowan (1930) has recently shown that liver extract 343 N.N.R., especially when given in conjunction with iron, promotes rapid regeneration of red blood-cells and hæmoglobin in fowls suffering from tape-worm anæmia, which suggests that under certain circumstances liver can promote erythropoeisis in the bird as it can in man. Barlow (1930) has failed to demonstrate any such influence on the anæmia of rice disease in pigeons. Until we know the exact bone-marrow findings in the latter condition it is impossible to explain these somewhat divergent findings.

WEIGHT.

It was found that the pigeons receiving the least pure preparation supplied by Dr. Edwin J. Cohn and Dr. Thomas L. McMeekin showed a pronounced gain in weight. The seven birds receiving this preparation showed in about 12 days an average weight gain of 38·4 gm., or 13·1% of their original weight, which varied from 292 gm. to 477 gm. The gain in weight on liver extract 343 N.N.R. by mouth was variable, as reference to Table II will prove. A gain in weight of patients with pernicious anæmia fed upon both whole liver and commercial extract has also been noted by Meulengracht and Holm (1929) and Vaughan (1930*a*). The significance of this weight gain is discussed elsewhere (Vaughan, 1930*b*). McGowan (1930) reports a similar weight gain in his anæmic fowls treated with liver extract No. 343 N.N.R.

PATHOLOGICAL FINDINGS.

Sections of tissues of the birds were examined microscopically, but the material is insufficient for drawing any definite conclusions. The bone-marrow of the birds given potent material effective in pernicious anæmia showed alterations from normal, suggestive of the changes which occur when liver is fed as an exclusive diet to pigeons (Muller, 1927 and 1929). This alteration

consists of a diminution of megaloblastic tissue and increase of white blood-cells, and thus resembles somewhat the change towards normal that occurs in patients with pernicious anæmia during remissions (Peabody, 1927).

DISCUSSION.

The administration of liver or its substitutes, as already noted, exerts its most pronounced therapeutic effects in anæmias associated with a megaloblastic bone-marrow. Presumably, therefore, the megaloblastic character of the pigeon's bone-marrow explains why liver preparations administered to these birds gives rise to a somewhat similar effect. Two questions at once arise: (1) How far is this effect on the pigeon's bone-marrow specific to substances effective in pernicious anæmia? and (2) What is the mechanism of their action in a presumably healthy bird?

SPECIFICITY OF SUBSTANCES EFFECTIVE IN PERNICIOUS ANÆMIA.

Preparation 132 EB2 and a subfraction, 132 ECB1, which were only guessed to be inactive, gave a positive reticulocyte response in pigeons. Leucine also gave a reaction in some preliminary experiments though it was inactive clinically, but no conclusions can yet be drawn from the results obtained with the latter preparation, which is being further investigated.

In view of these facts, it is not possible at present to state that those factors which are effective in pernicious anæmia exert an absolutely specific effect on the bone-marrow of the grain-fed pigeon. It is suggested, therefore, that there is possibly a group of substances very closely related to the principle effective in pernicious anæmia which are able to exert such a specific effect.

The mode of action of these substances on the hematopoietic organs of the pigeon may be variously interpreted. From previous work by one of us (G.L.M., 1929), in which whole liver was fed exclusively to pigeons, it was suggested that some substance in the liver when fed in excess was unfavourable to megaloblastic formation in the pigeon's bone-marrow. As a tentative explanation of the results described in the present paper, it seems possible that the grain-fed pigeon is suffering from a mild dietary deficiency, and owing to this, its marrow is in a state capable of responding by an increased delivery of young cells. Birds that come straight from the market often have reticulocytes in the vicinity of 18%, and it is only when they have been on a strict mixed grain diet and in confinement for some weeks that the reticulocyte count reaches a steady level of about 10%. This suggests that with their normal diet the reticulocytes are maintained at higher levels. Some support is given to such an hypothesis by the fact that it was possible to maintain a high reticulocyte count indefinitely with no alteration in the red-cell count or hemoglobin by the oral administration of liver extract No. 343 N.N.R. It must be remembered that the normal red cells of the pigeon are nucleated, and the bone-marrow in the grain-fed bird is megaloblastic, that is, the degree of development is less than in man. This state of affairs perhaps may account for the fact that, while, in the anæmic man, coincident with a change in the bone-marrow, the reticulocyte count rises and then falls to a low level, to be followed by a rise in mature red cells, in pigeons with a normal red blood-cell count the rise in reticulocytes is maintained.

CONCLUSIONS.

Experiments dealing with the administration of substances effective in pernicious anæmia and other test material to grain-fed pigeons are described:

1. The oral or intravenous administration of liver preparations effective in anæmia produced a consistent pronounced response of the reticulocytes, and one preparation and two subfractions prepared from it which had received no clinical tests also caused a similar response.

2. Beef-steak was as effective as potent liver preparations in causing a rise of the reticulocytes.

3. No reticulocyte response was obtained to (1) a liver preparation which was inert for pernicious anæmia, (2) a liver extract in which the active principle had been destroyed, (3) vitamin B 1 and 2, (4) histamine (5) sodium choridide, (6) casein.

4. An isolated observation suggests that sepsis interferes with the action of a potent liver preparation in pigeons as it does in pernicious anæmia.

5. Substances capable of influencing the reticulocyte level are without effect on the total red blood-cell count.

6. The grain-fed pigeon may perhaps suffer from a mild dietary deficiency, which is possibly a factor of importance in permitting its bone-marrow to respond to certain substances.

It is a pleasure to thank Anna Ryan for her valuable technical assistance throughout the investigation.

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VIRULENCE OF HÆMOLYTIC STREPTOCOCCI. II: THE INFLUENCE OF OXYGEN ON THE MAINTENANCE OF VIRULENCE IN BROTH CULTURES.*

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Received for publication September 12th, 1930.

Virulent hæmolytic streptococci may be attenuated by two distinct processes:

i. By natural, or artificial, selection of glossy variants, which are avirulent and deficient in type-specific substance; this variation is favoured by high oxygen tensions owing to the selective action of the peroxide synthesized by the streptococci (Todd, 1930).

ii. By a loss of virulence without any alteration in the morphology of colonies and without any loss of type-specific substance; this variation is dependent on an unknown factor which determines whether matt cultures equally rich in type-specific substance are virulent or attenuated. The invasive power of matt cultures is influenced by the oxygen tension at which the cocci are cultivated; high oxygen tensions favour maintenance of virulence, while low oxygen tensions cause loss of virulence. The following experiments were arranged to study matt virulent and matt attenuated cultures; alterations of virulence due to the appearance of glossy variants were, as far as possible, excluded.

Preliminary Observations on the Cultivation of Hæmolytic Streptococci in Aërated Broth.

Cultures in 5 c.c. of Douglas's tryptic digest broth were aërated by Ridley's mixing apparatus (1928), which was adjusted so that the cultures were alternately drawn up a tube, and expelled again, twelve times per minute, during the whole period of incubation. Cultivation under these conditions caused a rapid accumulation of peroxide, which became sufficiently concentrated, after 24 hours' incubation, to sterilize the culture. A rough estimate of the quantity of peroxide which accumulates in aërated broth cultures, after 24 hours' incubation, was made by preparing progressive dilutions of aërated culture in distilled water, and by determining the dilution in which the peroxide was insufficient to give a positive potato benzidine reaction (Avery and Morgan, 1924). Three drops of a saturated solution of benzidine in acetic acid were added to each dilution; small pieces of potato were then dropped into the tubes; and a blue coloration of the potato constituted a positive peroxide

* The expense of this research was partly defrayed by a grant from the Medical Research Council.

reaction. Dilutions of hydrogen peroxide (A.R. 20 vols.) were also made in distilled water and tested in the same way. Comparison of the results obtained in the two series of tests showed that a 1 in 16 dilution of aërated culture and a 1 in 3200 dilution of reagent hydrogen peroxide both gave faintly positive peroxide tests. The culture therefore contained peroxide equivalent to a 1 in 200 dilution of 20 vols. hydrogen peroxide A.R. (0·03% H_2O_2)—a concentration which is known to kill hæmolytic streptococci within a few hours.

The rapid auto-sterilization of hæmolytic streptococci in aërated broth cultures is demonstrated by the following experiment: Two tubes containing 5 c.c. of "unboiled" broth were sown with equal volumes of a matt virulent culture of the strain "Aronson Schnitzer"; they were incubated together, but while one of them had a small surface area exposed to the atmosphere by the ordinary aërobic method, the other was aërated by Ridley's mixing apparatus working at twelve cycles per minute. Samples, removed from the cultures at intervals, were tested for peroxide; and sterility tests were also made by subculturing 0·05 c.c. into 5 c.c. of broth and by plating one loopfull on blood-agar.

The aërated culture gave a positive peroxide reaction within 15 hours, it was almost sterilized in 23 hours (6 colonies on blood-agar), and it was completely sterilized in 38 hours; on the other hand, the aërobic culture was alive after 8 days' continuous incubation and it did not form any peroxide until the fourth day, when, owing to the removal of successive samples, the surface area exposed to the atmosphere was large in relation to the remaining volume of culture.

The Preservation of Peroxide in Different Batches of Broth.

Experiments were quoted in a previous communication (1930) showing that peroxide disappears from different batches of broth at different rates; in the following experiment the volume of oxygen given off by hydrogen peroxide in different batches of broth and in saline was measured.

As a preliminary experiment 1 part of hydrogen peroxide (A.R. 20 vols.) was added to 1000 parts of infusion broth prepared from horse-flesh and containing 2% Difco peptone. Two different batches of Douglas's tryptic digest broth, prepared from bullock's heart, were treated in the same way with hydrogen peroxide. The infusion broth was adjusted to pH 7·8 and the digest broth to pH 7·4. The three preparations were incubated at 37°C. and peroxide tests were done at intervals. The infusion broth and No. 2 digest broth both gave negative potato benzidine reactions after 4 hours' incubation; No. 1 digest broth gave a faintly positive reaction after 15 hours and a negative reaction after 24 hours.

The rate of oxygen liberation from reagent hydrogen peroxide in the three batches of broth was determined by adding 0·5 c.c. of hydrogen peroxide (20 vols.) to 5 c.c. of each batch of broth; the tubes were then sealed with heavy vaseline plugs and incubated at 37°C. A control tube containing 0·5 c.c. of peroxide and 5 c.c. of saline was included in the series, and all the tubes were immersed in boiling water for half an hour, to expel dissolved gases, before adding peroxide. The volumes of gas liberated, calculated from measurements

of displacement of vaseline plugs above the surface of the fluids, are shown in Fig. 1.

Gas appeared above No. 2 digest broth after 6 hours' incubation, above No. 1 digest broth after 24 hours, above saline after 5 days, and above infusion broth after 30 days. The curves show that the largest volume of gas was slowly liberated from peroxide in saline, and this was probably due to the absence of oxidizable substances. A smaller quantity of gas was liberated more quickly from both batches of digest broth; and although the preliminary experiment showed that peroxide disappeared equally quickly from infusion

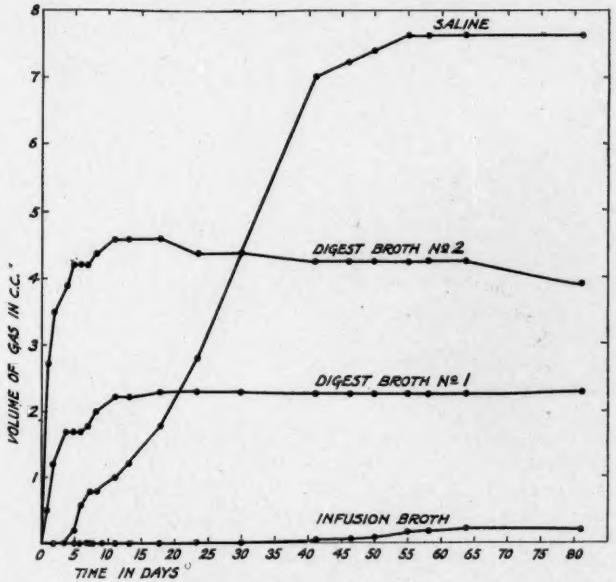
SALINE
DIGEST BROTH Nº 2
DIGEST BROTH Nº 1
INFUSION BROTH
VOLUME OF GAS IN C.C.
TIME IN DAYS
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
1 2 3 4 5 6 7 8

FIG. 1.

broth and from No. 2 digest broth, yet only a small bubble of gas was given off from the former medium after 81 days' incubation. This was presumably due to absorption of liberated oxygen by oxidizable substances in the infusion broth. After 81 days' incubation the fluids were tested for peroxide; No. 1 digest broth gave a strongly positive potato benzidine reaction, and all the other fluids, including the saline, gave negative reactions. No. 1 digest broth therefore possessed properties which preserved peroxide; and pH determinations showed that this preservation was not due to acidity.

The experiment shows that the rate of peroxide destruction varies in different batches of broth, that peroxide may break down more quickly in saline than in broth, and that there is considerable variation in the ability of different broths to absorb the oxygen liberated by destruction of peroxide. It

is not known why peroxide is preserved in certain batches of broth and rapidly destroyed in other batches of similar medium.

Accumulation of bacterial peroxide in aërated broth cultures of hæmolytic streptococci is probably largely influenced by two properties of the particular batch of broth used for cultivation: (*a*) ability to preserve peroxide, (*b*) reducing capacity.

Subcultivation of Virulent Hæmolytic Streptococci in Broth at Various Oxygen Tensions.

It has already been shown that large quantities of peroxide are formed in aërated broth cultures of hæmolytic streptococci; the following experiment was done to determine whether the peroxide, formed in aërated subcultures of virulent organisms, would exert a selective action and lead to the appearance of glossy variants. Matt virulent cultures of the strains "London" and "Aronson Schnitzer" were subcultured daily in aërated broth; after ten subcultures both strains remained matt and retained their original virulence, although each subculture of both strains had given strongly positive peroxide reactions. The observation that virulent hæmolytic streptococci are not easily reduced to the glossy state by subcultivation in aërated broth was utilized to investigate the effect of subculturing virulent organisms in broth under different oxygen tensions. Daily subcultures were made (i) in aërated broth, (ii) in aërobic broth, (iii) in anaërobic broth, and the virulence of the cocci for mice was tested at intervals. All the subcultures were made in 5 c.c. of Douglas's tryptic digest broth; the aërated cultures were continuously aërated throughout the period of incubation by Ridley's mixing apparatus working at twelve cycles per minute; the aërobic cultures were incubated in the ordinary way without any attempt to exclude, or to introduce, oxygen; and the anaërobic cultures were made in broth which was immersed in boiling water for half an hour, cooled rapidly in running water, immediately sown with cocci from the previous anaërobic culture and placed in a MacIntosh and Fildes jar without delay. Aërated cultures usually remained alive for 20 hours, and this period of incubation was therefore adopted as the daily routine for all cultures. Occasionally, however, aërated cultures were sterilized by peroxide before 20 hours' incubation had been completed, and they were therefore subcultured daily after 14 hours', 17 hours' and 20 hours' incubation to secure continuity of subculture.

Experiment A.—The matt virulent form of the strain "Aronson Schnitzer" (M.L.D.=10^{-7} c.c.) was used in this experiment. An ordinary aërobic broth culture which had been incubated for 16 hours was tested for virulence, and it was then subcultured into aërated broth, into aërobic broth and into anaërobic broth. Aërobic and anaërobic subcultivation was continued for 8 days and each subculture was incubated for 20 hours; the aërated subcultures were also continued for 8 days, but owing to occasional sterilization by peroxide the incubation periods were 17, 20, 14, 20, 20, 20, 20, 20 hours. Each subculture was tested for peroxide; all the aërated subcultures gave strongly positive potato benzidine reactions; on the other hand, the aërobic and the anaërobic subcultures all gave negative peroxide tests. Ordinary aërobic broth cultures were made from the eighth subcultures and tested for virulence after 16 hours'

incubation. The mice received, intraperitoneally, 0·5 c.c. of the respective cultures diluted in broth, and dilutions were also plated in blood-agar for colony counts.

TABLE I.—*Changes of Virulence Due to Subcultivation of Matt Virulent Cocci in Broth at Different Oxygen Tensions. Strain "Aronson Schnitzer."*

Dose of culture inoculated into mice (c.c.).	After 8 subcultures in—		
	Aërated broth.	Aërobic broth.	Anaërobic broth.
10^{-7}	†2 days	S	S
10^{-6}	†1 day	S	S
10^{-5}	†1 „	S	S
10^{-4}	†1 „	S	S
10^{-3}	†1 „	S	†14 days
10^{-2}	†1 „	†10 days	†1 day
10^{-1}	†1 „	S	†1 „
Average count of colonies from 10^{-7} c.c.	16	35	14

In all tables the following symbols are used: S indicates that the mouse was alive and in good condition one month after inoculation; † indicates death within the time stated.

Table I shows that virulence was maintained after eight subcultures in aërated broth; while the same number of subcultures in aërobic broth and in anaërobic broth caused a definite loss of virulence.

Subcultivation was continued and virulence tests were repeated after thirteen subcultures. The results of the second series of virulence tests were as follows: (1) Culture from aërated broth, smallest fatal dose = 10^{-8} c.c. (all the mice died within 2 days); (2) culture from aërobic broth, 10^{-3} c.c., died 10 days; 10^{-2} c.c., survived; 10^{-1} c.c. died within 24 hours; (3) culture from anaërobic broth, smallest fatal dose = 10^{-3} c.c. (all mice died within 24 hours).

The cultures used for virulence tests were plated on methæmoglobin horse-blood agar. The cocci derived from aërated subcultures formed typical matt colonies; those derived from aërobic and anaërobic subcultures also formed matt colonies, but their surfaces were covered with small papillæ.

The partially attenuated culture from aërobic broth was subcultured 14 times in aërated broth to investigate the possibility of increasing virulence by this method; peroxide was formed in each subculture, and the virulence of the organisms remained unchanged.

Experiment B.—In this experiment the strain "Dochez" (M.L.D. 10^{-6} c.c.) was used; the technique of Experiment A was repeated. Table II gives the results of virulence tests.

Fourteen subcultures in aërated broth and in aërobic broth did not appreciably alter the virulence of this strain. On the other hand, anaërobic subcultivation caused attenuation.

These changes of virulence were not associated with changes in colony form; all the colonies, including those of the final anaërobic subculture which had lost its virulence, were matt.

The attenuated culture from anaërobic broth was subcultured 14 times in

aërated broth; no peroxide was formed in any of the subcultures, and the virulence of the organisms remained unchanged.

TABLE II.—*Changes of Virulence Due to Subcultivation of Matt Virulent Cocci in Broth at Different Oxygen Tensions. Strain "Dochez."*

Dose of culture inoculated into mice (c.c.).	After 14 subcultures in—		
	Aërated broth.	Aërobic broth.	Anaërobic broth.
10^{-6}	S	+3 days	S
10^{-5}	+2 days	+1 day	S
10^{-4}	+9 „	+4 days	S
10^{-3}	+1 day	S	S
10^{-2}	+1 „	+1 day	S
10^{-1}	+1 „	+1 „	+1 day
Average count of colonies from 10^{-6} c.c.	83	220	434

Experiment C.—In this experiment the strain "London" was used; the broth of the aërated, aërobic and anaërobic subcultures was boiled each day immediately before subcultivation; otherwise the technique was the same as in Experiments A and B.

All the aërated subcultures, with one exception, gave positive peroxide tests; the aërobic and the anaërobic subcultures did not form peroxide. Subcultivation of this strain in aërobic broth and in anaërobic broth failed to cause any decrease of virulence, and it did not cause any alteration in the morphology of the colonies. After 19 subcultures in aërated broth the culture was composed of approximately equal numbers of matt and glossy colonies, and the result of a virulence test was indefinite, possibly due to the presence of a large number of avirulent glossy variants. It is possible that this strain is less sensitive to differences of oxygen tension than the strains "Aronson Schnitzer" and "Dochez," and that more prolonged anaërobic subcultivation might have evolved a matt attenuated culture. The experiment was stopped after 19 subcultures owing to the appearance of large numbers of glossy colonies in the aërated subcultures.

Changes in Appearance of Broth Cultures after Subcultivation at Different Oxygen Tensions.

In addition to differences shown by the virulence tests recorded in Experiments A and B there were also differences in the appearance of broth cultures. In Experiment A (strain "Aronson Schnitzer") the original culture formed a clumpy deposit at the bottom of the tube and the supernatant broth remained clear; on shaking it became uniformly turbid, but floccules reappeared after standing at room temperature for about an hour. This characteristic was maintained throughout the whole series of aërated subcultures. The anaërobic and the aërobic subcultures, however, lost their clumpy appearance after five or six subcultures, and then continued to form cultures of uniform turbidity with sediment lying in a uniform layer at the bottom of the tube; after

shaking sedimentation did not reappear for many hours. In Experiment B (strain "Dochez") the aërated subcultures showed an interesting alternation of clumpiness and uniform turbidity which appeared to be related to peroxide formation. This sequence of events is shown in Table III.

TABLE III.—*Showing the Time of Incubation, Naked-eye Appearance, Peroxide Production, and Virulence of Aërated Subcultures. Strain "Dochez."*

Subculture.	Time of incubation (hours).	Naked-eye appearance of cultures in broth.	Peroxide production.	Smallest fatal dose of aërobic cultures derived from each aërated subculture (c.c.).
1	17	Clumpy	++++	...
2	20	Uniform turbidity	0	...
3	14	Clumpy	0	...
4	20	,,	0	...
5	20	,,	+++	...
6	20	Uniform turbidity	0	10^{-6}
7	20	Clumpy	++++	10^{-6}
8	20	,,	++++	10^{-6}
9	21	General turbidity with a few clumps	0	10^{-4}
10	20	Clumpy	0	10^{-6}
11	20	,,	0	10^{-6}
12	21	,,	0	10^{-6}
13	21	,,	0	10^{-6}
14	20	,,	0	10^{-5}

Mice survived the following doses, which are larger than the doses recorded in the table:
From Subculture 7 = 10^{-5} c.c.
,, ,, 8 = 10^{-4} c.c.
,, ,, 13 = 10^{-4} c.c. and 10^{-5} c.c.
The remainder died within 2 days.

The first subculture was clumpy and produced abundant peroxide; the second subculture, after exposure to peroxide, was uniformly turbid and failed to produce any peroxide. In the third and fourth subcultures clumpiness reappeared, but peroxide formation was suspended till the fifth subculture. The second exposure to peroxide was immediately followed by complete loss of clumpiness and by disappearance of peroxide formation. After continued subcultivation the strain became stabilized in a clumpy form which had lost its function of peroxide formation. The fourteenth subculture was incubated for 70 hours in aërated broth and tested for peroxide at intervals, but even after this prolonged incubation peroxide did not appear. Changes in peroxide formation and of clumpiness were not associated with significant changes of virulence, but it is possible that uniform turbidity was due to the presence of virulent and attenuated cocci in the same culture. Clumpiness persisted in anaërobic broth up to the fifth subculture, when it permanently disappeared. The aërobic subcultures remained clumpy throughout the experiment. Peroxide tests on the aërobic and on the anaërobic subcultures were uniformly negative.

Anaërobic subcultivation of the strains "Aronson Schnitzer" and "Dochez" caused the organisms to form uniformly turbid broth cultures of attenuated virulence, but it did not cause the appearance of glossy variants.

The Influence of Peroxide on the Virulence of Hæmolytic Streptococci.

In Experiment A virulence was maintained after subcultivation in aërated broth, and peroxide was formed in each subculture. In Experiment B virulence was maintained under the same conditions of aëration, although the cocci lost their peroxide-producing function. In Experiment C the results were indefinite owing to the appearance of glossy variants in aërated subcultures.

If we exclude the natural selection of glossy variants by peroxide from consideration, these experiments leave room for doubt as to the possible effect of peroxide on the virulence of matt cultures. Does the accumulation of peroxide in aërated broth cultures favour the maintenance of virulence? Is virulence maintained in aërated broth if the accumulation of peroxide is prevented by catalase?

In an experiment designed to answer these questions virulent organisms were subcultured in aërated catalase broth; red blood-cells were used to supply the catalase, and they were separated from white cells and from serum by the following method: Freshly drawn human blood was passed through a tightly packed cotton-wool plug to remove the leucocytes as recommended by Fleming (1926); it was then centrifuged, and the red cells were washed six times with saline to remove serum. The saline was pipetted off after the final washing, and the packed cells were stored in the refrigerator. The addition of 0·01% of red cells to aërated broth cultures prevented the occurrence of positive potato benzidine reactions; half this quantity of cells did not prevent peroxide accumulation.*

To investigate the influence of peroxide on the maintenance of virulence, a virulent culture of the strain "Aronson Schnitzer" was subcultured eight times in aërated catalase broth and in aërated plain broth; eight subcultures were also made in aërobic catalase broth and in aërobic plain broth. In this experiment the maximum aëration which could be obtained with Ridley's mixing apparatus was used; the subcultures in aërated plain broth formed large quantities of peroxide, while the other subcultures gave negative peroxide tests. The results of virulence tests are given in Table IV.

Organisms from aërated catalase broth equalled, and possibly slightly exceeded, in virulence the cocci which had been exposed to peroxide in aërated plain broth. Organisms subcultured in aërobic catalase broth lost virulence, showing that catalase alone without aëration does not maintain virulence.

We have seen that a matt attenuated culture of the strain "Aronson Schnitzer" was not reverted to the virulent form by subcultivation in aërated plain broth, and it seemed possible that this failure of reversion might be due

* In experiments quoted in a former paper (1930) a sterile suspension of *Micrococcus Lysodeikticus* was used to supply catalase free from peroxidase. Penrose and Quastel (1930) have shown that this organism contains heat-stable peroxidase. They found that the peroxidase reaction, which may be obscured by the action of catalase, can be obtained if the catalase is first destroyed by heat.

to peroxide accumulation in the aërated subcultures. A matt attenuated culture of the strain "Aronson Schnitzer" (M.L.D. more than 0·1 c.c.) was therefore subcultured thirteen times in aërated catalase broth, and each subculture gave a negative potato benzidine reaction. The conditions of aëration were considered satisfactory, as a parallel series of cultures in aërated *plain* broth formed peroxide in each subculture. In order to show that the matt attenuated cocci could be made virulent by appropriate treatment, thirteen control subcultures were made in equal parts of normal horse-serum and broth. The results of this experiment are given in Table V. Subcultivation in normal serum caused a definite increase of virulence, but subcultivation in aërated broth did not alter the virulence of the cocci, although peroxide accumulation was prevented by catalase.

TABLE IV.—*Changes of Virulence Due to Subcultivation of Matt Virulent Cocci in Broth at Different Oxygen Tensions, Showing the Effect of Preventing Peroxide Accumulation by Catalase. Strain "Aronson Schnitzer."*

Dose of culture inoculated into mice (c.c.).	Original culture at commencement of experiment.	After 8 subcultures in—			
		Aërated plain broth.	Aërated catalase broth.	Aërobic plain broth.	Aërobic catalase broth.
10^{-8}	†3 days	S	†3 days	S	S
10^{-7}	S	†1 day	†2 „	S	S
10^{-6}	†2 days	S	†1 day	S	†9 days
10^{-5}	†1 day	†2 days	†1 „	S	S
10^{-4}	†1 „	†1 day	†1 „	†2 days	†3 days
10^{-3}	†1 „	†1 „	†1 „	†1 day	†1 day
10^{-2}	†1 „	†1 „	†1 „	†2 days	†1 „
10^{-1}	†1 „	†1 „	†1 „	†1 day	†1 „
Average count of colonies in 10^{-6} c.c.	66	211	288	351	323

TABLE V.—*Changes of Virulence Due to Subcultivation of Matt Attenuated Cocci (M.L.D. more than 0·1 c.c.) in Aërated Broth Containing Catalase to Prevent Peroxide Accumulation and in Equal Parts of Normal Horse-Serum and Broth. Strain "Aronson Schnitzer."*

Dose of culture inoculated into mice (c.c.).	After 13 subcultures in aërated catalase broth.	After 13 subcultures in equal parts of normal horse-serum and broth.
10^{-8}	S	S
10^{-7}	S	†3 days
10^{-6}	S	S
10^{-5}	S	†2 days
10^{-4}	S	†7 „
10^{-3}	S	†5 „
10^{-2}	S	†1 day
10^{-1}	S	†1 „
Average count of colonies in 10^{-6} c.c.	212	142

DISCUSSION.

It was shown in a previous paper (1930) that the natural selection of glossy variants by peroxide, in *agar* cultures, is dependent on two distinct factors: (*a*) the relative resistance of the glossy forms of certain strains to inhibition by peroxide, and (*b*) the early auto-sterilization of virulent colonies.

All the subcultures mentioned in the present communication were made in *broth*; and, as the aërated cultures were constantly mixed, the bacterial peroxide was uniformly distributed throughout the medium. The early auto-sterilization of virulent organisms was therefore prevented, and the relative resistance of glossy organisms to inhibition by peroxide was the only known factor tending to select glossy variants. It has already been pointed out that selective inhibition by peroxide is of minor importance, since peroxide does not accumulate till the end of the logarithmic phase of multiplication, when proliferation is minimal. This is probably the reason why glossy variants do not necessarily become predominant after subcultivation in aërated broth, although peroxide is formed in each subculture.

Virulence is maintained by subcultivation in broth at high oxygen tensions, and the effect of aëration is enhanced if accumulation of peroxide is prevented by catalase. On the other hand, anaërobic subcultivation causes the evolution of matt attenuated cultures, and prevents the appearance of glossy variants. Experiments A and B show that the strain "Aronson Schnitzer" is attenuated by anaërobic, and also by aërobic, subcultivation; on the other hand, the strain "Dochez," though readily attenuated anaërobically, is not affected to the same extent by subcultivation in aërobic broth. The rapid attenuation of the strain "Aronson Schnitzer" by subcultivation in aërobic broth may possibly be due to the lower oxidation-reduction potential attained by this strain when a limited supply of oxygen is available.

Organisms, attenuated by anaërobic subcultivation, form uniformly turbid cultures in broth, but they form matt colonies on solid media; it is therefore difficult to correlate the appearance of broth cultures with the different colony forms. Uniform turbidity of broth cultures is unreliable evidence of attenuation, since highly virulent matt cultures of the strain "London" have been obtained which form uniformly turbid cultures in broth. It is interesting to note that the character of broth cultures of matt attenuated organisms (strain "Aronson Schnitzer") depends on the method of attenuation; cultures attenuated by long-continued daily subcultivation on aërobic "unboiled" agar form granular deposits in broth, while cultures attenuated by anaërobic subcultivation form uniformly turbid broth cultures. Glossy cultures of this strain, obtained by subcultivation on "boiled" agar, also form granular broth cultures.

Comparison with previous experiments suggests that matt cultures, attenuated by anaërobic subcultivation, retain their full quota of type-specific substance; and this conclusion is supported by the observation that they are easily reverted to the virulent state by subcultivation in normal serum. Direct observations on type-specific substance, by Lancefield's technique, have not been made with the cultures described in this paper, as the strains "Aronson

Schnitzer" and "Dochez," which are easily attenuated by anaërobic subcultivation, are both exceptionally poor antigens for the production of type-specific antisera.

Matt attenuated cultures can be obtained from virulent cultures, either by anaërobic subcultivation, or by subcultivation on aërobic agar under certain conditions. Continued exposure to bacterial peroxide on agar ultimately causes loss of virulence, either by the natural selection of glossy variants, if a sufficiently large quantity of peroxide acts on the organisms for an adequate time, or by the evolution of a matt attenuated culture, if smaller quantities of peroxide act on the organisms during a long succession of short periods. This is illustrated by the observation that glossy variants are selected by subcultivation of virulent organisms on freshly "boiled" agar, while a matt attenuated culture is evolved after a much longer series of daily subcultures on "unboiled" agar.

Although virulence is fully maintained by subcultivation in aërated catalase broth, matt attenuated cultures are not reverted to the virulent form by this method of subcultivation. Possible reasons for this discrepancy will be discussed in a subsequent paper on the increase of virulence which occurs when matt attenuated organisms are subcultured in serum.

SUMMARY.

Virulent hæmolytic streptococci maintain their virulence after subcultivation in aërated broth, and they change to the matt attenuated form after subcultivation in anaërobic broth.

The effect of aëration is increased if peroxide accumulation is prevented by catalase.

Matt attenuated cultures are not reverted to the virulent form by subcultivation in aërated catalase broth.

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VIRULENCE OF HÆMOLYTIC STREPTOCOCCI. III: THE INFLUENCE OF OXYGEN ON THE RESTORATION OF VIRULENCE TO MATT ATTENUATED CULTURES.*

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Received for publication September 12th, 1930.

EXPERIMENTS will be described in this paper showing that the increase of virulence which occurs when matt attenuated hæmolytic streptococci are subcultured in normal horse-serum is dependent on the oxygen tension of the serum.

Undiluted normal horse-serum was heated at 56° C. for three hours to expel air. A tube of Douglas's tryptic digest broth was immersed in boiling water for half an hour to remove dissolved gases, and equal parts of heated serum and of boiled broth were set up immediately after the completion of heating, as follows: (1) 1 c.c. of 50% serum in a 4″ × $\frac{3}{8}$″ test tube was sealed with melted vaseline to exclude air; (2) 1 c.c. of 50% serum in a 4″ × $\frac{3}{8}$″ test-tube was not sealed, so that a small surface area was exposed to the atmosphere; (3) 1 c.c. of 50% serum was placed in a 50 c.c. Erlenmeyer flask so that a very shallow layer was formed with a large surface area exposed to the air.

A matt culture of the strain "Dochez," which had been partially attenuated by subcultivation in anaërobic broth was transferred to the three preparations of 50% serum, and daily subcultures were made into three similar preparations of freshly heated serum and broth. All the subcultures were tested for peroxide by the potato benzidine reaction with negative results. Sixteen-hour aërobic broth cultures were prepared from the 11th subcultures in 50% serum and tested for virulence with the results shown in Table I.

The graded aëration of the subcultures caused a corresponding grading of virulence; organisms from sealed tubes retained their original virulence; those from open tubes were more virulent than the original culture; and the most virulent organisms were derived from subcultures in Erlenmeyer flasks.

The experiment was continued; the cocci from flasks were subcultured daily in 50 c.c. Erlenmeyer flasks as before, and also in 100 c.c. Erlenmeyer flasks. All peroxide tests were negative up to the 25th subculture, when virulence was again tested, with the results shown in Table II.

* The expense of this research was partly defrayed by a grant from the Medical Research Council.

TABLE I.—*Changes of Virulence Due to Subcultivation in 50% Normal Horse-Serum at Different Oxygen Tensions. Strain "Dochez."*

Dose of culture inoculated into mice (c.c.)	Original culture at commencement of experiment.	After 11 subcultures in 50% serum in—		
		Sealed 4″ × ⅜″ tubes.	Open 4″ × ⅜″ tubes.	Erlenmeyer flasks.
10^{-6}	S	S	S	S
10^{-5}	S	S	S	†4 days
10^{-4}	S	S	†17 days*	†1 day
10^{-3}	S	S	† 6 „ ‡	†3 days
10^{-2}	†1 day	†1 day	† 1 day	†1 day
10^{-1}	†1 „	†1 „	† 1 „	†1 „
Average count of colonies from 10^{-6} c.c.	237	377	250	236

In all tables the following symbols are used: S indicates that the mouse was alive and in good condition one month after inoculation; † indicates death within the time stated; — indicates that the test was not done. The diluent was broth.

* *Post mortem* nothing abnormal detected; heart's blood sterile; sections of spleen normal.

‡ Heart's blood—pure culture hæmolytic streptococci.

TABLE II.—*Changes of Virulence Due to Subcultivation in 50% Normal Horse-Serum at Different Oxygen Tensions. Strain "Dochez."*

Dose of culture inoculated into mice (c.c.).	After 25 subcultures in sealed 4″ × ⅜″ tubes.	After 25 subcultures in open 4″ × ⅜″ tubes.	After 25 subcultures in 50 c.c. Erlenmeyer flasks.	After 11 subcultures in 50 c.c. Erlenmeyer flasks and 14 subcultures in 100 c.c. Erlenmeyer flasks.
10^{-8}	S	S	S	†2 days
10^{-7}	S	S	†7 days‡	S
10^{-6}	S	S	†2 „	†2 days
10^{-5}	S	S	†2 „	†2 „
10^{-4}	†18 days*	†2 days	†2 „	†1 day
10^{-3}	S	†1 day	†1 day	†2 days
10^{-2}	† 2 days	†1 „	†1 „	†1 day
10^{-1}	† 1 day	†1 „	†1 „	†1 „
Average count of colonies from 10^{-6} c.c.	259	155	142	147

* Heart's blood and spleen—pure culture of staphylococci; no streptococci found.

‡ Heart's blood—pure culture of hæmolytic streptococci.

Table II shows the same result as the earlier test: the most virulent organisms were again derived from the subcultures which received the largest supply of air.

The result of a third virulence test is shown in Table III: six mice were inoculated with 10^{-3} c.c. of a 16-hour broth culture from sealed tubes; and the same number of mice received 10^{-7} c.c. of a culture derived from Erlenmeyer flasks. Colony counts showed that the former dose represented 348,000 colonies, while blood-agar plates containing the latter dose developed an average of 22 colonies.

TABLE III.—*Changes of Virulence Due to Subcultivation in 50% Normal Horse-Serum at Different Oxygen Tensions. Strain "Dochez."*

	After 26 subcultures in 50% serum in—	
	sealed tubes. Dose of culture = 10^{-3} c.c.	open Erlenmeyer flasks. Dose of culture = 10^{-7} c.c.
	†1 day	†1 day
	†2 days	†1 ,,
	†2 ,,	†1 ,,
	S	†5 days
	S	S
	S	S
Colony counts	348,000	22

The organisms from Erlenmeyer flasks were ten thousand times more virulent than those derived from sealed tubes.

The cultures were plated on heated blood-agar after each of the ten virulence tests recorded in Tables I, II and III; they all formed indistinguishable typical matt colonies. There were, however, definite differences in the appearance of the broth cultures, the virulent cultures being more clumpy than the attenuated cultures.

THE RELATION BETWEEN VIRULENCE AND PEROXIDE FORMATION IN SERUM SUBCULTURES.

In the experiment described above the strain "Dochez" did not form peroxide in any of the subcultures, and the increase of virulence was directly proportional to the aëration of the subcultures. In the following experiment a matt attenuated culture of the strain "Aronson Schnitzer" was subcultured in 50% serum at various oxygen tensions, and all the Erlenmeyer subcultures gave strongly positive peroxide reactions, while the subcultures in sealed tubes and in open tubes gave negative peroxide tests. The formation of peroxide in this experiment was probably dependent on two factors: (1) The strain "Aronson Schnitzer" synthesizes more peroxide than the strain "Dochez." (2) In the first experiment, with the strain "Dochez," the serum was fresh and therefore contained a considerable quantity of catalase; in the second experiment, with the strain "Aronson Schnitzer," the serum was older and the catalase was partially oxidized.

Table IV shows that virulence was not increased by subcultivation in Erlenmeyer flasks when peroxide was formed.

The original culture was attenuated; subcultivation in sealed tubes and in open flasks caused no significant increase of virulence; on the other hand, subcultivation in open tubes caused a definite increase of virulence. It seemed probable that the peroxide formed in the Erlenmeyer flasks prevented an increase of virulence, and that the most favourable conditions for increasing virulence would be the greatest exposure to air compatible with the absence of peroxide formation. The experiment was therefore modified to obtain these conditions. Subcultivation in sealed and in open $4'' \times \frac{3}{8}''$ tubes proceeded as before and, in addition, cocci from the open tube were subcultured daily in

1 c.c. of 50% serum in open 7″ × 1″ tubes. The serum and the broth were heated each day to expel air, and all the subcultures gave negative peroxide reactions. Daily transfers in Erlenmeyer flasks were also continued, but the organisms in this line of subcultures were sterilized by peroxide before the second virulence test which is recorded in Table V.

TABLE IV.—*Changes of Virulence Due to Subcultivation of Matt Attenuated Cocci in 50% Normal Horse-Serum at Different Oxygen Tensions. Strain "Aronson Schnitzer."*

Dose of culture inoculated into mice (c.c.).	Original culture at commencement of experiment.	After 11 subcultures in 50% serum in—		
		Sealed 4″ × ⅜″ tubes.	Open 4″ × ⅜″ tubes.	Open Erlenmeyer flasks.
10^{-8}	—	S	S	S
10^{-7}	—	S	S	S
10^{-6}	—	S	S	S
10^{-5}	—	S	S	S
10^{-4}	S	S	†3 days	S
10^{-3}	S	S	†3 „	S
10^{-2}	S	S	†3 „	S
10^{-1}	S	†8 days	†3 „	S
Average count of colonies from 10^{-6} c.c.	21	51	48	46

TABLE V.—*Changes of Virulence Due to Subcultivation of Matt Attenuated Cocci in 50% Normal Horse-Serum at Different Oxygen Tensions. Strain "Aronson Schnitzer."*

Dose of culture inoculated into mice (c.c.).	After 20 subcultures in—		After 11 subcultures in open 4″ × ⅜″ tubes and 9 subcultures in 7″ × 1″ tubes.
	Sealed 4″ × ⅜″ tubes.	Open 4″ × ⅜″ tubes.	
10^{-8}	†20 days*	S	†3 days‡
10^{-7}	S	S	S
10^{-6}	S	S	†5 days
10^{-5}	S	S	†3 „
10^{-4}	S	† 2 days	†3 „
10^{-3}	S	† 8 „	†3 „
10^{-2}	S	†25 „ ‡	†2 „
10^{-1}	† 2 days	† 2 „	†1 day
Average count of colonies from 10^{-6} c.c.	13	18	43

* *Post mortem* encysted tape-worm in liver; heart's blood very anæmic, sterile.
‡ Pure culture hæmolytic streptococci from heart's blood.

Nine additional subcultures in sealed and in open 4″ × ⅜″ tubes did not alter the virulence of the organisms, but subcultivation in larger tubes, allowing the greatest oxygenation of the medium which could be obtained without peroxide formation, caused a definite additional increase of virulence.

THE MORPHOLOGY OF COLONIES AFTER SUBCULTIVATION IN SERUM.

In this series of experiments with the strain "Aronson Schnitzer," all the broth cultures used for virulence tests were identical in appearance and formed granular deposits surrounded by clear broth; yet examination of colonies on methæmoglobin horse-blood agar, which is the best medium for demonstrating the different colony forms of this strain, showed interesting variations which, so far as is known, are peculiar to the strain "Aronson Schnitzer." Virulent cultures formed large colonies with moderately matt surfaces; the matt attenuated culture, which was obtained after 126 subcultures on "unboiled" agar slopes, formed smaller colonies with intensely matt surfaces. Subcultivation in 50% serum reverted the smaller colonies to the virulent form, the degree of reversion apparently depending on the oxygen tension of the medium in which the cocci were subcultured.

Changes in colony form may be summarized as follows:

i	Original virulent culture	Colonies all one type	Large matt.
ii	After 126 subcultures on agar slopes	„ „	Smaller intensely matt.
iii	After 11 subcultures in Erlenmeyer flasks	„ „	„ „ „
iv	After 20 subcultures in small sealed tubes	„ „	Intermediate size less matt than ii.
v	After 20 subcultures in small open tubes	„ „	Intermediate size less matt than iv.
vi	After 9 subcultures in large open tubes	Two types of colonies in about equal numbers	(*a*) large matt (same as i); (*b*) intermediate size (same as iv).

The two colony forms which appeared after subcultivation in the large open tubes were separated by selecting colonies from a plate and their virulence was tested. A broth culture prepared from the smaller colonies was completely avirulent; a broth culture prepared from the large colonies was fatal in a dose of 10^{-8} c.c., but four mice which received larger doses survived. In spite of these discrepancies it is evident that virulent and avirulent cocci were both present in the same culture after nine subcultures in 50% serum in $7'' \times 1''$ tubes.

THE EFFECT OF HEATING SERUM AND BROTH.

A number of similar experiments have been done in which dissolved air was not removed from the serum and broth by heat immediately before subcultivation; and, under these conditions of relatively high oxygen tension, virulence can be fully restored by subcultivation in 50% serum in open $4'' \times \frac{3}{8}''$ tubes, and it can be partially restored by subcultivation in sealed tubes.

A number of experiments have also been done to see if virulent cultures can be attenuated by subcultivation in 50% serum under conditions which allow peroxide formation, and although sterilization by peroxide has invariably occurred before decisive results were obtained, the experiments seem to indicate that attenuation may occur if peroxide is formed in the serum subcultures.

These experiments show that the increase of virulence which occurs when matt attenuated cocci are subcultured in normal serum is dependent on the

aëration of the subcultures. If no steps are taken to remove oxygen from the serum and broth an increase of virulence occurs without any further absorption of oxygen from the air; if oxygen is removed by heat any increase of virulence is dependent on absorption of oxygen from the atmosphere, and is therefore influenced by the surface area of medium exposed to the air. It is, however, uncertain what part the serum plays in this process. Does it merely act as an efficient carrier of oxygen? Does it contain some substance which only becomes active as an augmentor of virulence in the presence of oxygen? Or does it act as a stabilizer which tends to maintain the medium at the optimum oxygen-tension necessary for increasing virulence when oxygen is available? Before discussing these questions experiments on constituents of normal serum deserve attention.

CONSTITUENTS OF NORMAL SERUM.

The Antibody to Bacterial Nucleo-protein.

Dawson and Avery (1927) found that rough pneumococci are reverted to the smooth virulent form by subcultivation in the serum of animals immunized with rough pneumococci. Dawson (1928) found that samples of normal serum usually contain traces of antibody to rough pneumococci, and that they occasionally contain sufficient antibody to cause reversion of rough pneumococci to the virulent form. Reimann (1926) showed that sera prepared with rough pneumococci are immunologically similar to sera prepared with pneumococcal nucleo-protein.

The following experiment was done to see if the effect of normal horse-serum on the virulence of hæmolytic streptococci is attributable to the small amount of antibody to streptococcal nucleo-protein (P) which it frequently contains. An attenuated culture of the strain "Aronson Schnitzer" was subcultured daily in digest broth containing 1% of high titre anti-P serum, and 0·01% of washed red blood-cells was added to prevent accumulation of peroxide. The cultures were aërated so that any increase of virulence caused by the anti-P might be maintained by aëration. The anti-P serum was prepared by inoculating rabbits with the purified nucleo-protein of hæmolytic streptococci.

Control subcultures were made in unheated 50% normal horse-serum in open $4'' \times \frac{3}{8}''$ tubes. The M.L.D. for mice at the beginning of the experiment was greater than 10^{-1} c.c.; after 11 subcultures in aërated catalase broth containing anti-P serum it remained unchanged, and after the same number of subcultures in 50% normal horse-serum it had changed to 10^{-5} c.c. This experiment seems to indicate that the ability of normal horse-serum to increase the virulence of hæmolytic streptococci is not dependent on the small quantity of anti-P which it contains.

Lysozyme.

Fleming and Allison (1927) have shown that non-hæmolytic streptococci may be made resistant to lysozyme by subcultivation in egg-white, and that cocci treated in this way are also resistant to intracellular digestion after phagocytosis by leucocytes. They suggest that virulence may be increased by the selection of lysozyme-resistant organisms. It seemed possible that an

increase of virulence after subcultivation in normal horse-serum might be due to the evolution of lysozyme-resistant cocci, as the normal horse-serum used in the experiments described in this paper contained a considerable quantity of lysozyme. The following experiments were therefore done:

A matt virulent culture of the strain S43 (M.L.D. 10^{-6} c.c.) was obtained by mouse passage; a matt attenuated culture of the same strain (M.L.D. 10^{-1} c.c.) was obtained by subcultivation on agar free from lysozyme; a glossy culture of the same strain (M.L.D. 10^{-1} c.c.) was obtained by subcultivation in immune rabbit serum which contained lysozyme and the homologous type-specific antibody.

(i) *The Resistance of the Three Cultures to Human Lysozyme and to Mouse Lysozyme.*

Equal volumes of the three cultures were inoculated into graduated dilutions of human tears in glucose broth. After overnight incubation the virulent cocci were alive in 25% tears, but they were sterilized in 50% tears; the matt attenuated and glossy cultures survived in 50% tears, but were sterilized in 70% tears. The avirulent cultures were therefore more resistant to human lysozyme than the virulent culture.

Mouse lysozyme was prepared by placing the liver, spleen, kidneys and heart of a mouse in filtered acetone by an aseptic technique; the acetone was then removed by evaporation at 37° C., and the dried organs were extracted with normal saline at room temperature. Tests with *M. lysodeikticus* on agar plates showed that the extract contained a considerable quantity of lysozyme, but a titration was impossible because the extract was turbid.

Equal volumes of the three cultures were sown in equal volumes of extract, and blood-agar counts showed that both the matt cultures were completely sterilized after 22 hours' incubation; on the other hand, the glossy organisms multiplied 100 times in 22 hours. The glossy avirulent culture was far more resistant to mouse lysozyme than either of the other cultures.

(ii) *The Resistance of the Three Cultures to Phagocytosis.*

Phagocytic experiments with human blood and with mouse blood gave the same results; the glossy cocci were more easily phagocyted than either of the other cultures.

(iii) *The Resistance of the Three Cultures to the Bactericidal Action of Blood.*

The bactericidal action of defibrinated human blood was tested on the three cultures. All the organisms planted in blood were killed within three hours, but the time required for complete sterilization in human blood was graded according to the virulence of the organisms for mice; the glossy culture was sterilized first, and the virulent culture was sterilized last.

The bactericidal action of defibrinated mouse blood was also tested. All the cultures multiplied in the pooled blood of several mice, but the rate of multiplication agreed with the results of virulence tests; the virulent culture multiplied most rapidly while the glossy culture multiplied most slowly.

(iv) *Rate of Multiplication in Normal Serum.*

Normal human serum and normal rabbit serum were used in these experiments, and they both contained considerable quantities of lysozyme. The glossy culture multiplied more rapidly in both sera than the matt cultures.

These experiments show that an avirulent culture may be more resistant to lysozyme than a virulent culture of the same strain, and that lysozyme resistance may be increased and virulence diminished in one process by subcultivation in serum which contains both lysozyme and type-specific antibody. They show also that matt virulent cultures are not necessarily more resistant to lysozyme than matt attenuated cultures, and that virulence is increased by mouse passage without increasing the resistance of the organisms to lysozyme. We may therefore assume that the increase of virulence for mice which occurs when matt attenuated hæmolytic streptococci are subcultured in normal horse-serum is not due to the evolution of a lysozyme-resistant culture.

DISCUSSION.

Experiments described in previous papers show that although the virulence of hæmolytic streptococci is diminished when the organisms are subcultured daily on agar, yet this loss of virulence does not occur if peroxide accumulation is prevented by catalase.

Observations on the pathogenicity of virulent cocci after subcultivation in broth at different oxygen tensions showed that virulence is lost under anaërobic conditions, that it is maintained if the organisms are liberally supplied with air, and that the effect of aëration is enhanced if accumulation of peroxide is prevented by catalase.

In conformity with these observations it was found that the increase in virulence of matt attenuated organisms after subcultivation in normal serum is directly proportional to the supply of air. Each increase in aëration causes an increase of virulence until a point is reached at which peroxide is formed, when the effect of aëration is reversed, and further increases of air supply cause attenuation.

Experiments with agar, with broth and with serum all show that virulence is favoured by high oxygen tensions if accumulation of peroxide is prevented; divergent results are, however, obtained with serum cultures, in which virulence is not only maintained, but increased, and with aërated catalase broth cultures, in which virulence is maintained but not increased. Evidence has been quoted showing that the action of serum is not due to the antibody of bacterial nucleoprotein (P) or to lysozyme, and a suggestion has been put forward that it is due to the oxygen-carrying capacity of serum. This theory receives confirmation from the work of Hewitt (1930), who followed the oxidation-reduction potential-time curves of cultures of the strain "Aronson Schnitzer" in media prepared from the broth and the serum which were used in the experiments on virulence recorded in this paper. He found that cultures in aërobic broth reached a negative potential of $-0{\cdot}16$ volt, and that cultures in 50% serum did not reach a lower Eh than $+0{\cdot}05$ volt. Hewitt's curves, which show the oxygen-carrying capacity of serum, explain why virulence is maintained in

serum cultures, as the electrode potential-time curves of cultures in 50% serum and in aërated catalase broth are almost identical. There is, however, no satisfactory explanation of failure to increase the virulence of matt attenuated organisms by subcultivation in aërated catalase broth. Observations have been made on the pH of cultures in 50% serum and in aërated catalase broth, but no significant differences were found. Possibly serum is effective because it poises the Eh during all phases of growth at, or near, the optimum level for increasing virulence; on the other hand, mechanical aëration would probably require periodical adjustment in order to reproduce the conditions of oxygenation which are automatically attained by serum; intense aëration being provided during the logarithmic phase, when the organisms have the greatest reducing action, followed by aëration of diminishing intensity as the cocci pass into the resting phase. It is also possible that the different results obtained with aërated catalase broth and with 50% serum may be due not so much to differences in the Eh of the media as to differences in respiratory exchange between the media and the organisms.

It is of interest to note the stability of virulence in a constant environment; an attenuated culture is rendered more virulent after a few subcultures in 50% serum at a moderate oxygen tension, but continued subcultivation in serum at the same oxygen tension does not cause an additional increase of virulence. A similar phenomenon was noted in experiments on the attenuation of virulent organisms by subcultivation in anaërobic broth; a virulent culture of the strain "Dochez" (M.L.D. $= 10^{-7}$ c.c.) was attenuated by subcultivation in anaërobic broth; after 10 subcultures the M.L.D. was 10^{-2} c.c.; after 20 subcultures, and again after 90 subcultures, the M.L.D. remained unchanged (10^{-2}). This seems to show that hæmolytic streptococci rapidly adjust their virulence to the oxygen tension of their environment, when no further change of virulence occurs as long as the oxygen tension of the medium remains constant. These observations raise interesting points for speculation on the possible relationship between the virulence of an infecting organism and the oxygen tension of the tissues of the host.

SUMMARY.

The increase of virulence which occurs when matt attenuated cultures of hæmolytic streptococci are subcultured in 50% normal horse-serum is dependent on the oxygen tension of the serum.

The optimum condition for increasing virulence is the highest oxygen tension which can be obtained without peroxide formation.

The effect of normal serum on virulence appears to depend on its oxygen-carrying capacity.

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TUBERCULIN SENSITIVITY IN RATS.

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Received for publication October 30th, 1930.

IN a previous paper (1927) the authors reported that sensitiveness to tuberculin had not developed in rats infected with tubercle bacilli, and at the same time fed on a diet lacking fat-soluble vitamins A and D. Eight weeks after infection the animals had been given intraperitoneal injections of Frankfort standard tuberculin equal to 0·35 c.c. of the undiluted fluid. No difference in reaction was observed between groups of rats infected or not infected with tubercle bacilli, or of infected rats fed or not fed with the fat-soluble vitamins; the animals survived the injections. Subsequently, Smith (1928), pushing the dosage to lethal limits, was able to show that rats on a diet lacking fat-soluble vitamins were much more sensitive to "tuberculo-protein" when they were infected with tubercle bacilli than when they were not. The "tuberculo-protein" used by this worker consisted of a water-soluble substance obtained from cultures of *B. tuberculosis* grown on a synthetic non-protein medium; it was given intravenously.

A repetition of this method of testing for sensitivity was performed by the authors, maintaining, however, the use of old tuberculin injected intraperitoneally for the test dose. The dietetic technique was that described in their previous paper.

Experiment 1.—Rats were intraperitoneally infected, each with 5 mgm. of a young glycerin-agar culture of the human tubercle strain H37, kindly supplied by Dr. Maurice I. Smith, 25 days after being placed upon a diet lacking fat-soluble vitamins. Five weeks after infection they were tested along with control uninfected rats, which had been for a similar period on the same deficient diet. The tuberculin employed was several times the strength of Frankfort standard tuberculin. Deaths, when they occurred, took place almost regularly within 24 hours, and therefore, to exclude any that might not be strictly attributable to the toxin, observation was limited to this period.

The results are given in Table I, and confirm the increased sensitivity to the lethal effects of tuberculin which is possessed by the rats as reported by Smith.

TABLE I.

Tuberculin per 100 gm. body-weight.	Rats uninfected with *B. tuberculosis*.	Rats infected with *B. tuberculosis*.
2·0 c.c.	2 out of 6 die	2 out of 2 die.
1·5 c.c.	3 „ 17 „ (18%)	11 „ 19 „ (58%)
1·0 c.c.	0 „ 4 „	0 „ 4 „

* Member of the Scientific Staff, Medical Research Council. Working with grants for assistance and expenses.

From these results it seems clear that rats infected with tubercle bacilli have less resistance to the toxic effects of tuberculin than have uninfected rats.

Previous to this test 9 of the infected rats and 8 of the uninfected rats had received 0·1 c.c. tuberculin intracutaneously in the shaven belly, and been under observation for 2 days, when a second and similar injection was made and the animals observed for a further 2 days. No difference between the two groups was noticed, and nothing indicative of a positive reaction seen in any of the animals, nor did post-mortem examinations of the tuberculous rats which succumbed to intraperitoneal injections of tuberculin reveal lesions suggestive of those presented by true cases of tuberculin shock.

In order to test the specificity of the lethal action of tuberculin in tuberculous rats, it was decided to try the effect of toxic substances derived from bacteria other than *B. tuberculosis*, and for this purpose a suspension of agar-grown *S. paratyphi A* was prepared and killed by heating at 60°C. for 1½ hours.

Experiment 2.—Rats which had been intraperitoneally infected with 5 mgm. of H37, 10 days after being placed on the fat-soluble deficient diet, were tested, along with uninfected controls also on the deficient diet, as to their resistance to this killed *Salmonella* suspension, 26 days after infection. A dosage of 10,000 million per 100 gm. body-weight was chosen and administered intraperitoneally. Table II gives the result of the experiment.

TABLE II.

Killed *S. paratyphi A* per 100 gm. body-weight.	Rats uninfected with *B. tuberculosis.*	Rats infected with *B. tuberculosis.*
10,000 million	7 out of 13 die (54%)	10 out of 13 die (77%)

This experiment is an indication that infection with tubercle bacilli has rendered rats more sensitive to the toxic substances of a *Salmonella* culture, just as it had in the previous experiment to those of a culture of *B. tuberculosis.*

To strengthen the evidence the experiment was repeated.

Experiment 3.—Here rats which had been inoculated intraperitoneally with 5 mgm. of H37, 3 weeks after having been placed on the fat-soluble deficient diet, were tested, along with the usual controls, for their resistance to a killed *S. paratyphi A* suspension, 29 days after infection.

Table III gives the results of the experiment in which, after a preliminary trial with the dosage of 10,000 million, the majority of the rats received 8000 million per 100 gm. body-weight.

TABLE III.

Killed *S. paratyphi A* per 100 gm. body-weight.	Rats uninfected with *B. tuberculosis.*	Rats infected with *B. tuberculosis.*
10,000 million	2 out of 4 die	3 out of 4 die
8,000 million	7 „ 16 „ (44%)	13 „ 16 „ (81%)

The results are similar to those given by Experiment 2, and although the numbers in each experiment are small, the increased sensitivities, occasioned

in each case by infection with *B. tuberculosis*, may be compared, if allowance be made for the fact that the test dose was of a different order of toxicity in each case.

Taking the critical amount of 1·5 c.c. tuberculin per 100 gm. body-weight, one sees in Experiment 1 that 18% of the uninfected and 58% of the infected rats succumb to the tuberculin; that is to say, out of 82 uninfected animals which would survive injection with this amount of tuberculin, 40 would have died if the rats had been infected with tubercle bacilli, which represents an acquired sensitivity of 50%. In Experiment 2, 54% of the uninfected and 77% of the infected succumb to the toxic substances of the *Salmonella* inoculum; that is to say, out of 46 uninfected animals which would survive injections with *S. paratyphi A* suspension, 23 would have died if the rats had been infected with tubercle bacilli, which represents an acquired sensitivity of 50%. In Experiment 3, 44% of the uninfected and 81% of the infected die after inoculation with 8000 million killed *Salmonella*; that is to say, out of 56 uninfected animals which would survive injection with this amount of killed *S. paratyphi A*, 37 would have died if the rats had been tuberculous, which represents an acquired sensitivity of 66%.

One may say that infection with tubercle bacilli has enhanced the sensitivity of these dietetically deficient rats to the toxins of a tubercle culture and to those of a *Salmonella* culture to no very dissimilar extent.

It is of interest to note here that according to Hirayama (1930), infection of the guinea-pig with *B. tuberculosis* does not bring about hypersensitivity to a heterologous toxin (in his experiments diphtheria toxin was used), but, on the contrary, an increased resistance. The guinea-pig suffers true infection by *B. tuberculosis*, whereas the rat does not. Tubercle bacilli, it is well known, may multiply in the tissues of the inoculated rat and occasion proliferation of certain of the tissue-cells, but invasion by the bacteria never results in the formation of tubercles, let alone caseation.

CONCLUSION.

Rats placed upon a diet deficient in fat-soluble vitamins A and D become more sensitive to the lethal effects of big doses of tuberculin after infection with *B. tuberculosis*, but a similar sensitivity is displayed by the tuberculous rat towards the toxic effects of a killed *Salmonella* suspension. The response is, therefore, not to be regarded as a specific one.

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A METHOD FOR THE KATAPHORESIS OF BIOLOGICAL MATERIALS.

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Received for publication November 6th, 1930.

THE apparatus to be described was designed to fulfil the following conditions:

1. Continuous path of buffer solution for the current passing between the electrodes, so that no agar bridges need be employed.
2. Uniformity of bore of the tube connecting the vessels containing the electrodes, so that the potential gradient across the moving boundary can be easily measured.
3. Ease of filling and forming a sharp boundary with small volumes (2 or 3 c.c.) of the suspensions to be tested.
4. Ability to withdraw whole sample should it be needed for other investigations.
5. Provision of a method of obtaining small samples during cataphoresis without disturbing the boundary, so that effect of reversal of polarity could be studied when boundary is invisible (*e. g.* virus suspensions).
6. The apparatus must be easy to wash out with cleaning fluids, etc.

The apparatus is shown in the diagrams (Figs. 1 and 1A). A, A and H, H are filled with buffer solution of the desired pH. Tap L is turned to place H, H in connection with I, and tap M to put the Hg reservoir K in connection with I. Strong NaCl solution is run in slowly from C, C to cover electrodes B, B. K is adjusted so that the Hg meniscus is brought to K_1. E, E_1 are left open and the apparatus left for about an hour for temperature and hydrostatic effects to disappear.* Tap L is now shut and M turned so that N and I are in connection. By turning L to J, the space I and J can be filled with Hg. By lowering N the sample is brought in through J, the Hg meniscus being brought back *exactly to the point* K_1. L is shut and M turned to K. On turning L to connect I and H, H, no movement of the meniscus at K_1 should be detectable. The boundary is formed as follows: A slow stream of water is run into tube K. This will cause a very slow rise in the meniscus at K_1. The rate is very easily controlled by the water entering K. The

* It was found that if the solutions to be employed were kept near the apparatus at room temperature some hours before use, it was not necessary to leave the apparatus overnight to come into equilibrium as described by many workers.

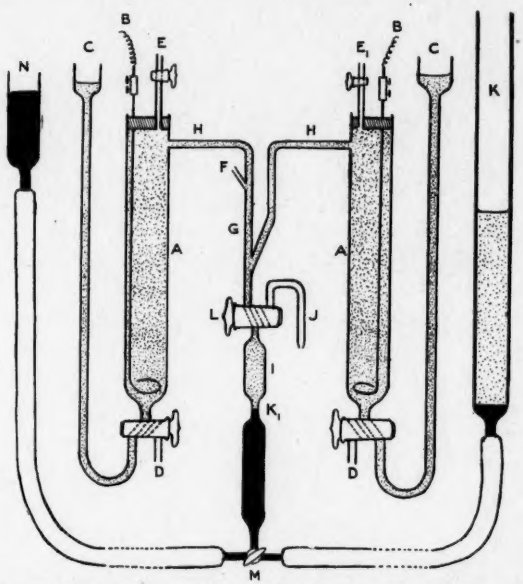

FIG. 1.—A, A. Electrode vessels (1 in. by 6 in.). B, B. Silver electrodes, paraffin waxed except ring on bottom. C, C. Tube to introduce buffer, and then strong NaCl to cover electrodes. D, D. Drainage-tubes for washing, etc. E, E_1. Taps. When E_1 is closed the right half of the apparatus is rigid and a sample can be withdrawn from F without disturbing the boundary. H, H. Tube of uniform bore, 3 mm. in diameter. K. Tube 24 in. long, the level of which is adjustable. It contains mercury to height of 2 or 3 in. When tap L is open to H, H and M to K_1 the mercury in K is level with the meniscus in K_1.

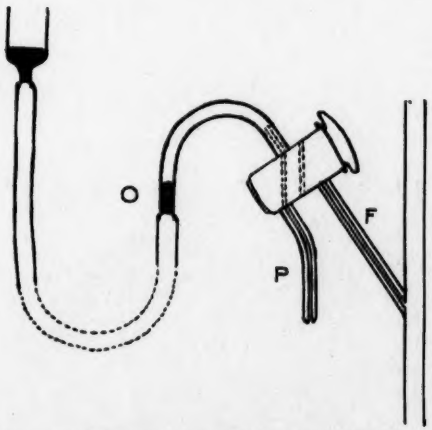

FIG. 1A.—F is connected to a two-way tap (1 mm.) by one of its limbs. The common limb is connected to a mercury reservoir. From O to F it is filled with B.P. paraffin oil. The sample is ejected by the third limb P.

boundary (which must be perfectly sharp) should be about half-way up the limb G in 30 minutes. L is now closed and the current may be turned on.

A milliammeter is included in the circuit and not more than 1 milliamp. should be passed. The potential gradient obtained in H, H is of the order of 5 volts per cm.

To sample during an experiment, the current is turned off and tap E_1 closed. The mercury reservoir shown in Fig. 1A is lowered so that about 0·2 c.c. is drawn into the common limb of the tap. The tap is then turned and the sample ejected at P. An equal volume of buffer is replaced through E. The polarity is reversed and kataphoresis continued. After a suitable interval a second sample should be negative.

SUMMARY.

1. An apparatus for the kataphoresis of biological materials is described.
2. No agar bridges are employed; a continuous path of buffer solution is present between the electrodes.
3. A new method of forming a sharp boundary is described.
4. Provision is made for withdrawing samples during kataphoresis without disturbing the boundary.

Our thanks are due to Dr. W. A. M. Smart for the preparation of the drawings.

DIPHTHERIA TOXIN-ANTITOXIN FLOCCULES.

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Received for publication November 6th, 1930.

In a previous paper (Marrack and Smith, 1930) we brought forward evidence that diphtheria toxin-antitoxin floccules were composed of denatured pseudoglobulin, and that the amount of precipitate obtained from a given antitoxic serum when mixed with an equivalent amount of toxin was independent of the conditions under which precipitation took place, particularly of the amount of non-specific protein present. From this we inferred that antitoxin is pseudoglobulin. The present paper consists of further experiments on the same subject.

I. INDEPENDENCE OF NON-SPECIFIC PROTEINS.

In our previous experiments the amount of protein which precipitated with the floccules was never more than one-ninth of the total protein of the mixture even when pseudoglobulin solutions were used. It might be argued that sufficient non-specific protein was present in any case to saturate the absorptive power of the toxin-antitoxin precipitate, and that this was the reason why further additions of non-specific protein made no difference to the total protein in the precipitate.

We have since received from the Wellcome Research Laboratories an antitoxic serum (3018) containing 8·1 mgm. of protein and 2300 units per 1 c.c. From this we prepared a pseudoglobulin solution by half saturation and dialysis which contained 1160 units and 27·3 mgm. protein per c.c.; one-third of the protein in this solution precipitated on adding an equivalent amount of toxin. Even with this antitoxin solution the addition of non-specific proteins did not affect the amount of floccule nitrogen per unit of antitoxin (Table I). These experiments were performed as described in the previous paper; the mixtures were frozen, and nitrogen estimated both with the spectroscope and by the micro-Kjeldahl method.

TABLE I.—*Effect of Non-Specific Protein on Amount of Protein in Floccules.*

Antitoxin added to 25 c.c. toxin = 350 Lf. units.	Non-specific protein added.	Total protein in mixture in mgm.	Floccules: total protein in mgm.		Floccules: γ* nitrogen per unit.
			Spectrometric.	Kjeldahl.	
0·3 c.c. pseudoglobulin 3018	0	8·25	3·66	3·64	16·1
Ditto	5 c.c. normal horse-serum	368·0	3·66	3·68	16·3
0·15 c.c. serum 3018	0	12·7	...	3·40	15·1

* 1γ = 0·001 mgm.

II. BEHAVIOUR OF ANTITOXIN AND FLOCCULES WITH PRECIPITINS FOR HORSE PSEUDOGLOBULIN.

Eisler (1920) has shown that the antitoxin of anti-tetanus horse-serum is removed by a precipitin for horse-serum. Similar work has been done with other antibodies; particularly Landsteiner and Prasek (1911) have brought forward convincing evidence that agglutinins for red blood-corpuscles and typhoid bacilli are proteins precipitable by antisera. Recently, however, Moloney and Weld (1925) have claimed that the titre of diphtheria antitoxin serum (horse) is unaltered by precipitation with an anti-horse-serum, whether this be prepared by injection of normal horse-serum or antitoxic serum. The explanation of such results and of the instances mentioned by Landsteiner and Prasek (1911), in which agglutinin was incompletely precipitated, is probably found in the work of Dean and Webb (1926): If the ratio of antigen/antibody exceeds that at which optimal precipitation occurs the precipitate may be voluminous, but considerable antigen may be left in the

supernatant fluid. We have made a series of experiments on the behaviour of diphtheria antitoxin (horse-serum) with the serum of rabbits immunized against normal horse-serum.

(A) *Removal of Antitoxin by Antiglobulin Serum.*

Experiment 1.—0·8 c.c. of a 1 : 25 dilution of antitoxic pseudoglobulin solution 3018 were mixed with 1·7 c.c. of 0·9% NaCl solution and 1·5 c.c. of the serum of rabbit 284, which had been immunized against normal horse pseudoglobulin; this mixture, which contained horse pseudoglobulin and antiglobulin serum in approximately optimal proportions, was frozen solid and kept in the ice-chest overnight. Next day it was thawed and centrifuged. The supernatant fluid was pipetted off and tested:—

(1) For antitoxin *in vitro*: To a series of tubes each containing 0·5 c.c. of the supernatant were added 0·35, 0·3, 0·25, 0·2, 0·15 c.c. of a toxin containing 14 units per c.c., and 0·9% NaCl solution to make the volume up to 0·85 c.c. The tubes were mixed and incubated at 45° C. for 6 hours, and kept in the ice-chest overnight. No cloudiness developed in any of them.

Since 0·5 c.c. of the original mixture contained 0·1 c.c. of the 1 : 25 dilution of pseudoglobulin 3018, and 0·1 c.c. of such a 1 : 25 dilution flocculated readily when mixed with 0·4 c.c. of 0·9% NaCl and 0·32 c.c. (the equivalent amount) of toxin, the supernatant must have lost well over a half of its antitoxin content.

(2) For antitoxin *in vivo*: To 1 c.c. of the supernatant fluid were added 0·1 c.c. of toxin containing 14 units per c.c. After the mixture had stood 2 hours 0·2 c.c. of it were injected intra-cutaneously into a guinea-pig; definite necrosis appeared at the site of injection in 3 days, but the guinea-pig survived. Some antitoxin must have remained in the supernatant, but a large part had disappeared since the 1 c.c. of the original mixture contained enough antitoxin to neutralize 0·64 c.c. of the toxin.

Experiment 2.—This was carried out as Experiment 1. The precipitating mixture contained 0·8 c.c. of the 1 : 25 dilution of pseudoglobulin 3018 and 1·0 c.c. of the serum of rabbit 298, the optimal proportions of globulin and antiserum. The final dilution of antitoxic pseudoglobulin solution in this mixture was 1 : 45 (0·3 c.c. of the solution diluted 1 : 45 with 0·9% saline flocculated rapidly with 0·29 c.c. of toxin containing 20 Lf. per c.c.). The mixture was treated as in Experiment 1 and the supernatant tested.

(1) *In vitro*: No opacity appeared when 0·3 c.c. of the supernatant was incubated at 43° C. with from 0·3 c.c. to 0·1 c.c. of toxin. The supernatant therefore contained considerably less than one-third the antitoxin added to the original mixture.

(2) *In vivo*: To 0·2 c.c. of the supernatant were added 0·02 c.c. of toxin (20 Lf. per c.c.). After standing in the ice-chest overnight the mixture was injected into a guinea-pig (weight 190 gm.); the animal was found dead on the third day after injection; the antitoxin content was therefore under one-ninth of that in the original mixture.

The supernatant fluid was also tested for precipitin by adding further pseudoglobulin as done by Dean and Webb; about one-twentieth of that in the original mixture was found to be still present.

(B) *Fixation of Toxin by the Precipitate Formed by Antitoxic Serum and Anti-pseudoglobulin Serum.*

Experiment 3.—The precipitate from Experiment 1 above, washed 3 times, was shaken with 2·5 c.c. of toxin (Lf. 14) and kept in the ice-chest overnight. It was then centrifuged and the supernatant fluid tested for toxin.

(1) *In vitro*: A series of tubes containing 0·4 c.c. of the supernatant were incubated at 43° C. with 0·0024, 0·002, 0·0017, 0·0011 and 0·0007 c.c. of antitoxin 3018; no flocculation nor cloudiness appeared in any tube. Since 0·4 c.c. of the original toxin was balanced by 0·0025 c.c. of antitoxin 3018, at least three-quarters of the toxin had therefore been removed from the supernatant.

(2) *In vivo*: 0·2 c.c. of the supernatant diluted 1 : 100 was injected intradermally into a guinea-pig; no lesion appeared. The toxin had therefore practically completely disappeared.

Experiment 4.—The precipitate from Experiment 2 was treated as above with 2 c.c. of toxin (Lf. 20). Of the supernatant 0·3 c.c. gave no cloudiness on incubation with 0·0027, 0·002, 0·00135 and 0·0009 c.c. of antitoxin 3018. Intracutaneous injection of 0·1 c.c. into a guinea-pig produced no lesion. So that according to the flocculation test over two-thirds, and according to the skin test all, the toxin had been removed.

Experiment 5.—In order to make sure that toxin was not fixed by the precipitate formed by normal horse pseudoglobulin and a precipitin, 1·4 c.c. of a 0·095% solution of normal horse pseudoglobulin was mixed with 1 c.c. of the serum 298B of a rabbit immunized against horse pseudoglobulin (optimum proportions), and the mixture was frozen solid. The precipitate was separated and washed as before, then stirred and repeatedly shaken with 2 c.c. of toxin (20 Lf. units per c.c.) and kept in the ice-chest overnight. Next day the supernatant was centrifuged off, and 0·3 c.c. portions tested with 0·0031, 0·0025, 0·0022, 0·0018 and 0·0009 c.c. of antitoxin 3018 (2300 units per c.c.). The tube contained 0·0025 c.c. of antitoxin flocculated first, so that no significant amount of toxin had been adsorbed by the precipitate.

(C) *Precipitation of Toxin-Antitoxin Floccules by Antipseudoglobulin Serum.*

Experiment 6.—To 25 c.c. of toxin (= 350 Lf. units) were added 0·15 c.c. of antitoxin 3018 making a balanced mixture, which was frozen solid and kept in the ice-chest overnight. Next day the floccules were centrifuged off, washed three times with 0·9% NaCl and dissolved with 6 c.c. of N/1000 acetic acid. This solution (pH 3·5) was heated at 60° C. for one hour, cooled, and the pH brought to 7·6. The cloudy solution was treated with antipseudoglobulin serum 284 (1) as shown in Table II. When human serum diluted 1 : 10 were used in place of serum 284 (1) no flocculation took place.

These experiments show conclusively that antitoxin and toxin-antitoxin floccules react like pseudoglobulin with antipseudoglobulin serum. The theory that antibodies are not proteins but are adsorbed by protein precipitates and adsorb protein when specific precipitation takes place involves considerable difficulties. In the first place it does not account for the quantitative relations that we have found (Marrack and Smith, 1930). In the second

TABLE II.—*Precipitation of Solution of Toxin-Antitoxin Floccules by Serum 284(1) containing Precipitin for Horse Pseudoglobulin.*

Floccule solution	0·5	0	0	0	0	0	0·5	0
Floccule solution diluted 1:10 with 0·9% NaCl	0	0·5	0·3	0·2	0·1	0·05	0	0·5
0·9% NaCl	0	0	0·2	0·3	0·4	0·45	0·5	0·5
Serum 284(1) diluted 1:10	0·5	0·5	0·5	0·5	0·5	0·5	0	0
After one hour at room temperature	Heavy floccules	Granular	Granular	Fine granules	Haze	Haze	Haze	Slight haze.
After night in ice-chest	Heavy precipitate	Precipitate	Precipitate	Precipitate	Slight precipitate	Slight precipitate	No precipitate	No precipitate.

place it does not agree with the facts that although antibodies are carried down when the proteins of the antiserum are precipitated by non-specific methods as by ammonium sulphate, or by specific methods by a precipitin, yet when, as found by Landsteiner and Prasek (1911), a protein precipitate is formed in an agglutinating goat-serum by adding foreign proteins (*e.g.* horse-serum) and precipitating them with a corresponding precipitin (*i. e.* serum of a rabbit immunized with horse-serum), the antibodies of the agglutinating serum are not carried down. The antibodies appear, therefore, to be associated only with the proteins of the serum in which they are developed and this association is quantitative.

Since antitoxin, when precipitated by a precipitin, can still combine with toxin, different groups must be involved in the two reactions.

III. RELATION TO ACID-BINDING GROUPS OF ANTIBODY.

(A) *Effect of Immunity Reactions on pH.*

It is possible that the acid- or base-binding groups of antigen and antibody are involved when combination occurs. This idea is supported by the observation by Hirsch (1922) of a change of pH in immunity reactions (a precipitin reaction and agglutination of bacteria), such as would occur when a weak acid formed an insoluble compound with a strong base. However, Hirsch seems to have taken no steps to ensure that his antigens were of the same pH as his antibody, nor to avoid changes of pH due to extraneous causes during the reaction. The changes he found were improbably large. According to Landsteiner and Prasek (1911), the antibody only forms about 1/800 of the total protein in an agglutinating serum; if all the acid-binding groups in this antibody protein were abolished it would not produce such a large change of pH in the proteins of the whole serum; this does not take into account the other buffers—bicarbonate and phosphate—of the serum.

To test fairly whether a change of pH accompanies an immunity reaction, it is necessary that the reacting substances should not be swamped by other buffers. These conditions are fulfilled when the antitoxic pseudoglobulin 3018 is mixed with purified toxin.

Experiment 7.—250 c.c. of toxin TX 502 (20 Lf. units per c.c.) were precipitated with acid in the cold. The precipitate was centrifuged off and

dissolved with 0·1 N. NaOH. The pH of the solution was 6·89 and volume 2 c.c.; 1 c.c. contained 1·7 mgm. nitrogen and 680 Lf. units (2·5 γ nitrogen per Lf. unit). To 2 c.c. of antitoxic pseudoglobulin 3018 (1130 units per 1 c.c.) were added 0·1 c.c. of 2 N. NaCl solution, and 0·13 c.c. of 0·04 N. NaOH to bring the pH to 6·85. 1·3 c.c. of this pseudoglobulin solution were added to 2 c.c. of the toxin solution covered with neutral paraffin oil in a small hard glass tube, which was then stoppered. The mixture became very thick at once. The pH immediately after mixing and after three-quarters of an hour was 6·86. The mixture was then frozen solid and re-warmed. It was then completely flocculated; the pH remained 6·86. After keeping overnight in the ice-chest the pH was still the same.

The buffer power of the supernatant fluid in this experiment was approximately twice that of the floccules. The pH estimations were accurate to within 0·02, so that the pH change in the reacting protein must have been under 0·06.

(B) *Iso-electric Point of Toxin-Antitoxin Floccules.*

If either acid-binding groups alone or base-binding groups alone were involved in the combination of toxin and antitoxin, some change in iso-electric point would probably result.

When toxin-antitoxin floccules are re-dissolved with acid, clearing usually begins about pH 4, although the suspension at about pH 8 is turbid. It appears, therefore, that re-solution occurs nearer to the iso-electric point of normal pseudoglobulin on the acid than on the alkaline side. This suggests that the iso-electric point of the floccules may be on the alkaline side of that of normal pseudoglobulin. A moderate shift in this direction was found by McCutcheon *et al.* (1930) in agglutination reactions. We have, therefore, measured the iso-electric point of the floccules, using an apparatus specially designed to give a very sharp boundary. This apparatus is described separately (Smith and Marrack, 1930); we need only mention here that no agar bridges, which are theoretically inadmissible in kataphoresis apparatus—see Maver (1928)—were used.

Combination of toxin and antitoxin apparently does not occur near pH 5; on the other hand, floccules in suspension when brought to this pH sink so rapidly that they are unsuitable for kataphoresis experiments. We therefore mixed toxin and antitoxin at pH about 7, allowed to stand for 20 minutes and adjusted to the required pH. To avoid the precipitation of euglobulin, antitoxic pseudoglobulin solution 3018 was used, and purified toxin was used owing to the high conductivity of the crude solution.

Experiment 8.—100 c.c. of toxin TX 502 were precipitated with acid in the cold; the precipitate centrifuged off, washed with distilled water, dissolved with the minimum amount of NaOH and made up to 10 c.c. This solution was titrated with antitoxic pseudoglobulin solution 3018.

An acetate buffer was made up with a sodium acetate concentration approximately 0·015 N., and run into the kataphoresis apparatus. Then to 2 c.c. of the toxin solution 0·3 c.c. of the antitoxin solution were added, forming a balanced mixture; this mixture was allowed to stand 20 minutes at room temperature. Sodium acetate solution was then added to make the salt

concentration equal to that of the buffer, and the pH measured with a glass electrode brought to that of the buffer with dilute acetic acid. The mixture, which became turbid at once, was run into the apparatus. Care was taken throughout to keep the fluids at the same temperature. Very sharp boundaries were obtained. The current varied from 0·45 to 0·5 milliamps., and the potential gradient was 5 volts per cm. In all cases the movement of the particles was reversed on reversing the current. At the end the suspension was run out and the pH again measured and was found unchanged.

The results are shown in Table III. It was not possible to make measurements between pH 5·1 and 5·6 as the floccules settled too rapidly. The iso-electric point of the floccules is therefore close to that found by Rona and Michaelis (1910) for normal pseudoglobulin (pH 5·4); it is not so alkaline as that found by McCutcheon *et al.* (1930) for sensitized bacteria.

As a matter of fact, owing to the greater steepness of the titration curve on the acid side, the degree of ionization on which solution may well depend is nearly as great at pH 4 as at pH 8.

TABLE III.—*Kataphoresis of Toxin-Antitoxin Floccules.*

pH.	Direction.	
4·91	To kathode	0·25 cm. in 30 mins.
5·09	„	Approximately 0·05 cm. in 15 mins.
5·61	To anode	„ 0·05 „ 15 „
5·74	„	0·20 cm. in 30 mins.

IV. HEAT OF REACTION OF TOXIN AND ANTITOXIN.

Bayne-Jones (1925), using a thermopile method, found that 0·06 calories were emitted when a unit of antitoxin reacted with an equivalent amount of toxin. The evolution of an amount of heat of this order would provide a convenient method of following the course of the reaction and studying its nature. But the amount is surprisingly large, being greater than that obtained by the complete metabolism of protein in the body, if our estimate of the amount of protein per unit antitoxin is correct, and still higher if our estimate is too high.

Since toxin can be obtained in solution many times more concentrated than the crude preparation and antitoxin is already very concentrated, it is possible to test this question of heat evolution with the simplest apparatus. A solution of toxin containing 500 Lf. units per c.c. such as we have used in the experiment below should, according to Bayne-Jones, evolve 30 calories in the reaction with antitoxin, and therefore raise the temperature of the mixture approximately 24° C. We have found no evidence of any heat evolution at all in the reaction; this does not disprove Arrhenius' (1907) estimate, but shows that no evolution of the order found by Bayne-Jones occurs.

Experiment 9.—250 c.c. of toxin TX 502 (20 Lf. units per c.c.) were precipitated with acid in the cold. The precipitate was centrifuged off, dissolved in 0·1 N. NaOH, and made up to 5 c.c. with distilled water plus sufficient 2N. NaCl solution to bring the final NaCl concentration to 0·1 N. This solution was titrated with antitoxic serum 3018 and found to contain 500 Lf. units per c.c. 4·3 c.c. of toxin solution and excess of antitoxic serum were

kept in separate test-tubes in a Thermos flask with strips free of silvering on opposite sides to allow for observation. In a control flask were placed test-tubes containing 4·3 c.c. of 0·9% NaCl solution and a similar amount of antitoxic serum. Both flasks were stoppered with rubber bungs through which passed thermometers graduated in one-tenths of a degree. When sufficient time had been allowed for temperature equilibrium to be reached, 0·95 c.c. of antitoxin 3018 (2300 units per c.c.) were added to the toxin solution and the thermometer bulb placed in the mixture, which was stirred. Immediately the control was treated in the same way. The toxin-antitoxin mixture became very turbid at once and flocculated in under three-quarters of an hour. The readings of the thermometers agreed throughout.

We have performed a similar experiment using horse-serum pseudoglobulin as antigen and a precipitin for it as antibody. In this case flocculation occurred within one minute; if heat were evolved in the process it would be more easy to detect it than when the process took about three-quarters of an hour.

Experiment 10.—This experiment was carried out as before. In one flask 4·0 c.c. of a solution of euglobulin prepared from serum B of rabbit 297, which had been highly immunized against horse pseudoglobulin, was mixed with 1·76 c.c. of 0·38% horse pseudoglobulin solution; this mixture contained antigen and antibody in optimum proportions. In the other flask 4·0 c.c. of 0·9% NaCl solution were mixed with 1·76 c.c. of the pseudoglobulin solution. Flocculation occurred within a minute, but no difference of temperature between the two mixtures was detected, at once or in the next half an hour.

The amount of antibody protein precipitated in this experiment (35 mgm.) was greater than in Experiment 7 (23 mgm.). Evolution of something like the same order of heat might be expected in both. This amount cannot exceed 0·02 calories per mgm. of antibody protein flocculated, and must be much less than 0·06 calories per unit in the case of union of diphtheria toxin and antitoxin.

CONCLUSION.

These experiments give further support to our contentions that diphtheria antitoxin consists of slightly modified pseudoglobulin molecules, and forms the greater part of toxin-antitoxin floccules. There is no evidence that acid or base-binding groups are involved in the flocculation process, or that any appreciable amount of heat is evolved.

SUMMARY.

1. The amount of protein in the floccules obtained from balanced mixture of diphtheria toxin and antitoxin is unaffected by the addition of non-specific proteins, even when a special antitoxin solution, of which one-third was precipitated in the floccules, was used.

2. Diphtheria antitoxin (horse-serum) is precipitated by an antiserum to horse pseudoglobulin; the precipitate so formed combines with diphtheria toxin.

3. No change of hydrogen ion concentration occurred when purified and concentrated toxin combined with a concentrated and specially pure antitoxin solution.

4. The iso-electric point of diphtheria toxin-antitoxin floccules lies between pH 5·1 and 5·6.

5. The heat of reaction of diphtheria toxin and antitoxin is not of the order found by Bayne-Jones (1925).

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OBSERVATIONS ON THE VIRUS OF PSITTACOSIS.

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Received for publication November 12th, 1930.

THE observation that psittacosis is due to a virus which is filtrable and which cannot be cultivated by methods suitable to the ordinary bacteria (Bedson, Western and Levy Simpson, 1930) has now been amply confirmed (Krumwiede, McGrath and Oldenbusch, 1930; Levinthal, 1930; Elkeles, 1930; Gordon, 1930; Sacquépée and Ferrabouc, 1930; Rivers and Berry, 1930). In a report of an investigation of an extended series of cases of this disease—both human and avian—reference was made to some preliminary observations bearing on the nature of the virus of psittacosis and some of the immunological problems concerned (Bedson and Western, 1930). The writing of that report coincided with the disappearance of the epidemic (May), and since that time we have had the leisure to study some of the strains of virus isolated. Our findings form the subject of this paper.

STRAINS ISOLATED.

Human Strains.

Previously the results of the investigation of 22 human cases were reported (Bedson and Western, 1930); from 14 of these a filtrable virus was isolated, 2 were negative, and in 6 doubtful findings were obtained. Since then 4 additional human cases have been examined. The material employed was citrated blood, and from 1 specimen a filtrable virus was isolated. Thus out of a total of 26 human cases examined, evidence of the presence of a filtrable virus has been obtained in 15, 6 gave doubtful findings and 5 were negative. An analysis of the findings obtained with various human material is given in Table I:

TABLE I.

Material.	Number of cases.	Positive.	Doubtful.	Negative.
Citrated blood	19	9	5	5
Serum	2	2	0	0
Pleural exudate	1	1	0	0
Cerebro-spinal fluid	1	0	0	1
Spleen	3	1	2	0
Liver	1	1	0	0
Lung	1	1	0	0
Pooled organs (liver, lung, brain)	1	1	0	0
,, (spleen, liver, lung)	1	0	1	0

In connection with the examination of human material, there are two features which are of particular interest. The first of these relates to the use of mice in the isolation of psittacosis virus direct from man. The susceptibility of the mouse is now well established (Krumwiede, McGrath and Oldenbusch, 1930; Gordon, 1930*a*; Rivers, Berry and Rhoads, 1930; Bedson and Western, 1930), and Gordon (1930*b*) and Rivers, Benjamin and Berry (1930) have found that this animal can be utilized for the demonstration of the virus in human material. This latter observation we are able to confirm, for in two instances mice have been infected direct from human cases by means of blood collected in an equal part of 2% sodium citrate in saline, the amount inoculated being 1·0 c.c. of the citrated blood (0·5 c.c. intraperitoneally and 0·5 c.c. subcutaneously). In both instances there was a considerable interval between the inoculation of the mice and their becoming ill—15 days in one case and 20 days in the other—a point which Gordon (1920*b*) has drawn attention to. The other point of interest concerns the keeping of human strains. Many of the strains isolated by us were discarded after the first positive passage, but an attempt was made to keep four alive in animals (budgerigars and mice). All these four strains have been lost after varying periods of animal passage, and none of them showed that tendency to increase in virulence when passed in mice which is a feature of many parrot strains. A similar experience has been encountered by Gordon (1930*b*).

Avian Strains.

In a previous communication (Bedson and Western, 1930) it was reported that 7 sick parrots associated with human cases had been examined and a filtrable virus isolated in every case. Since then 2 further parrots have been investigated with positive findings in 1, making a total of 9 birds examined, in 8 of which the presence of a filtrable virus was demonstrated. We have also had the opportunity of examining sick birds of other varieties than the parrot, associated with human disease diagnosed as psittacosis on clinical grounds. Two of these birds were canaries, both from separate sources, and from one of these a filtrable virus was isolated which was pathogenic for mice, producing changes in that animal characteristic of psittacosis. This strain was lost. Two other strains have been isolated, 1 from budgerigars and 1 from a Java sparrow. These birds all came from the same aviary in which an outbreak of psittacosis had occurred; 2 human cases were infected from this source. The budgerigar strain (G.B.) was isolated in mice from the pooled spleens of 3 birds and rapidly established itself. It has been carried to the 10th generation in mice. The Java sparrow strain (J.S.) was isolated from the pooled organs of 1 bird and proved as good as the budgerigar strain; it has been passed through mice for 13 generations. These strains are referred to not only because of their origin, but because they have been utilized together with our parrot strain P. XV in the experimental work recorded in this paper. Strain P. XV has now reached its 24th generation in mice.

PSITTACOSIS IN THE GUINEA-PIG.

In some earlier experiments (Bedson and Western, 1930) an attempt was made to establish psittacosis virus in the guinea-pig by intradermal inoculation in the plantar skin. This was unsuccessful. Further experiments on these lines have proved equally unfruitful. The guinea-pig, however, is susceptible, for the inoculation of psittacosis virus in the shaved hairy skin gives rise to a local reaction very similar to that evoked by herpes virus. Twenty-four hours after inoculation a raised, slightly red papule appears, which varies in size according to the amount of virus introduced. By the 48th hour the lesion has become larger, redder, and the centre in many cases is becoming necrotic. The papule then gradually recedes, the necrotic portion becoming a dry black scab; no vesicles are formed. This reaction in the guinea-pig's skin provides a most satisfactory means for titrating psittacosis virus. Our experience would suggest that the guinea-pig's skin is a less sensitive indicator than the mouse; even so a dilution of 1/10,000 of virulent mouse spleen will not infrequently give a reaction. Unless heavily inoculated in the skin, the guinea-pig shows no signs of a generalized infection, but in the case of an animal which has received multiple skin inoculations, refusal of food, staring coat and lethargy indicate a generalization of the infection. In our experience no such cases have ended fatally.

As a means of establishing this virus in the guinea-pig, intra-testicular inoculation is more satisfactory. Starting with a 5% suspension of virulent mouse spleen inoculated intratesticularly in the guinea-pig in a dose of 0·3 c.c.,

our P. XV strain has been carried through six serial passages in this animal species. Passage was made on the 2nd to 3rd day after inoculation, when the testicular lesion is usually at its height, a 10–20% suspension of testicle in phosphate being used for the purpose. The virus content of the testicle, although increasing with passage, never reached a high figure. Titrations made in the guinea-pig's skin with suspensions of 2nd and 4th generation testicle gave titres of 1/10 and 1/500 respectively. The last guinea-pig inoculated in this series—6th generation—died 48 hours after inoculation and showed injection and some enlargement of the inoculated testicle. The spleen was normal, the liver was dark in colour, the suprarenals injected and the lungs congested. Microscopically the lesions were in keeping with a generalized psittacosis infection. The liver showed marked congestion and degenerative change in the parenchyma, particularly towards the centre of the lobule. The spleen showed congestion and hæmorrhage in the pulp with a proliferation of reticulo-endothelium in that situation, and the lungs showed numerous small early pneumonic patches with dilatation of vessels, thickening of the alveolar walls, commencing desquamation of alveolar cells, and a mononuclear infiltration particularly in the neighbourhood of vessels. A mouse inoculated with a 10% suspension in phosphate of the pooled organs (lung, liver, spleen and testicle) of the guinea-pig became ill on the 4th day, and died on the 7th day. The post-mortem findings were those usual to psittacosis in the mouse, and since the tissues were culturally sterile the death of the animal was undoubtedly due to psittacosis virus.

FILTRABILITY.

In our earlier work it had been established that the virus was able to pass through Chamberland L 1 *bis*, L 2 and Seitz EK. filters, and although it was obvious that filtration removed a considerable proportion of the virus, we had no accurate quantitative observations on this point. A series of filtration experiments have been made in which the quantity of virus in the suspensions before and after filtration was determined by titration in the skin of the guinea-pig. The findings are given below (Table II) and require no further description.

CENTRIFUGATION OF THE VIRUS.

Although it is recognized that the filtrability of the virus through porcelain filters is not necessarily a criterion of its size, the results just given do suggest that the virus particles are relatively large. Experiments were made to see whether this virus could be thrown down by centrifugation. In these experiments Chamberland L 1 *bis* filtrates were used, and the virus titrations were made in the skin of the guinea-pig; in other respects the technique was identical with that employed in similar experiments made with herpes virus (Bedson, 1927). The results given in Table III indicate quite clearly that the filtrable forms of this virus can be thrown down by centrifugal forces, and this finding, taken in conjunction with the quantitative filtration experiments, indicates that the psittacosis virus particles are of a relatively large size, or else that the virus is absorbed to tissue particles of relatively large size.

TABLE II.

Strain of virus.	Material.	Filter.	Titration. Before. 1/20.	1/100	1/1000.	1/10,000.	1/100,000.	After. 1/20.	1/100.	1/1000.
Parrot XV, 9th gen.	Mouse spleen in M/50 phosphate pH 7·6	Chamberland L1 *bis*	...	+	+	±	−	+	±	?
,, 10th ,,	Ditto	Ditto	...	+	±	−	−	+	±	−
,, 6th ,,	,,	Chamberland L2	++	+	±	∓	−	+	±	−
,, 7th ,,	,,	Ditto	...	+	±	−	−	±	∓	−
,, 9th ,,	,,	Chamberland L3	...	+	+	±	−	?	−	−
,, 10th ,,	,,	Ditto	...	+	±	−	−	−	−	−
Budg. "G" 5th ,,	,,	Seitz EK.	++	+	±	−	−	±	−	−
Parrot XV, 11th ,,	Mouse spleen in broth	Chamberland L2	...	++	+	∓	−	+	±	−
,, 11th ,,	Mouse spleen in M/50 phosphate pH 7·6	Ditto	...	++	+	±	−	±	−	−
,, 18th ,,	Mouse spleen in broth	Chamberland L1 *bis*	++	+	±	∓	−	±	∓	−
,, 18th ,,	Mouse spleen in M/50 phosphate pH 7·6	Ditto	++	+	±	∓	−	±	?	−

Dilutions are those of spleen in phosphate.

TABLE III.

Material.	Titration. Before centrifugation. 1/20.	1/200.	1/2000.	1/20,000.	After centrifugation for 2 hours at 5000 r.p.m. Supernatant. 1/20.	1/200.	1/2000.	1/20,000.	Deposit. 1/20.	1/200.	1/2000.	1/20,000.
Mouse spleen in M/50 phosphate pH 7·6. Filtered Chamberland L1 *bis*	++	+	±	−	∓	−	−	−	++	+	+	±
Ditto	+	±	−	−	?	−	−	−	++	+	±	∓

IDENTIFICATION OF VIRUS WITH "BODIES" SEEN IN VIRULENT MATERIAL.

There is a considerable consensus of opinion that virulent psittacosis material contains small cocco-bacillary bodies which can be seen with the microscope (Levinthal, 1930; Lillie, 1930; Coles, 1930; Bedson and Western, 1930). These have been seen both in material from natural infections in man and parrots and in that coming from the experimental disease (birds and mammals). These bodies comprise small coccal forms occurring singly and in pairs, and bacillary forms which show a tendency to polar staining. They resemble in some respects the *Rickettsiæ*, and Lillie (1930) has gone so far as to suggest for them the name *Rickettsia psittaci*. On the other hand, apart from the bacillary forms, their resemblance to the Paschen bodies seen in vaccinia and variola material is quite close. They stain readily with Giemsa, and are particularly well brought out by differentiation with an orange G.-tannin solution (orange G., 1 gm.; tannic acid, 5·0 gm.; water, 100 c.c.). They can also be stained by one of the polychrome blues such as borrel blue, and can be demonstrated in sections stained with eosin-methylene blue or eosin-orange-toluidine blue. It has to be admitted that their presence in virulent material, more particularly of avian and human origin, is not constant, or at any rate, their numbers may be so small as to render their demonstration a matter of considerable difficulty, but in the tissues of the infected mouse not only are these bodies present in large numbers, but we have never failed to find them. Furthermore, an examination of a considerable number of mouse spleens would suggest that the number of bodies present varied with the virulence. The inability to demonstrate these bodies in all virulent material is not a sound argument against their being the virus. The same phenomenon has been observed in connection with the presence of *R. prowazecki* in virulent typhus material from different sources, and other examples might be cited. We are not in a position to say that these bodies are the virus, but the filter and centrifuge experiments reported above would be in keeping with the virus being of such dimensions. Coles (1930) considers that he has demonstrated the smallest coccal forms in virulent filtrates, and some observations made by us support this contention. Further, the shift in virulence produced by centrifugation would appear to be accompanied by a similar shift in the number of bodies, but since these experiments were made with filtrates containing only the smallest coccal forms, satisfactory evidence on such a point is not easy to obtain.

SURVIVAL OF THE VIRUS.

In glycerol.—In earlier work (Bedson and Western, 1930) it had been found that parrot organs preserved in 50% glycerol in the refrigerator at 6°C. maintained their virulence for at least three weeks. This does not appear to be a satisfactory method of storing virulent mouse spleens, for even a few days' sojourn in 50% glycerol-phosphate will frequently render the tissue avirulent. It is much more satisfactory to place the tissue in the buffered phosphate alone and keep in the cold.

Freezing.—Previous work had suggested that freezing tissues was a good way of conserving their virulence. The following experiment on this point supports this conclusion. A portion of the spleen of a mouse dying from psittacosis was suspended in phosphate to give a 1 in 20 suspension. The virulence of this suspension for mice was tested. The remainder of the spleen was kept frozen, its virulence for mice being tested from time to time by making a 1 in 20 suspension in phosphate and inoculating it intraperitoneally in a dose of 0·5 c.c. The results are given below (Table IV). In two other cases spleens kept frozen for 56 and 57 days were found to be highly virulent.

TABLE IV.

Time of testing.	Number of mice inoculated.	Result.
Immediately	2	Both killed moribund in 2 days.
7 days	2	One died 6th day. One very ill, recovered.
22 „	1	Died 6th day.
36 „	1	Killed moribund 7th day.

In phosphate.—It was of interest to see how rapidly a suspension of virulent tissue in M/50 phosphate pH 7·6 lost virulence when stored in the cold (6°C.). Should such a suspension maintain a constant virulence for any considerable length of time it would obviously be a great convenience. In the experiments given below (Table V) the virulence was titrated in the shaved skin of the guinea-pig as already described. The findings recorded in the table require no elaboration.

IMMUNOLOGICAL PROBLEMS.

Immunity Conferred by Experimental Infection.

It has been reported (Bedson and Western, 1930) that budgerigars which had recovered from infection had acquired a certain degree of immunity. The same has been found in mice.

Neutralizing Power of Convalescent Serum.

Experiments made in budgerigars (Bedson and Western, 1930) had given indefinite results. Other experiments made in mice and guinea-pigs have been negative. For instance: Equal parts of convalescent serum and virus

TABLE V.

Nature and strength of phosphate suspension.	When done.	Titration : Result.* 1/1.	1/10.	1/100.	1/1000.	1/10,000.
Mouse spleen, 5%	Immediately	+	±	∓	−	...
	7 days	++	+	±	−	...
	15 „	++	+	±	∓	...
	23 „	+	±	∓	−	...
	34 „	++	+	±	−	...
	43 „	∓	−	−	−	...
„ 10%	Immediately	...	++	+	?	−
	52 days	+	?	−	−	−
„ 5%	Immediately	...	++	+	±	−
	55 days	+	?	−	−	−
„ 5%	1 day	++	+	±	?	−
	57 days	±	∓	−	−	−
	68 „	∓	?	−	−	−
„ 5%	2 „	++	+	±	∓	−
	64 „	±	−	−	−	−
	75 „	−	−	−	−	−

* The dilutions are of the original suspension.

suspensions containing 50 and 5 guinea-pig skin doses of virus respectively were mixed and allowed to stand at room temperature for 2 hours, controls being put up with a normal human serum. At the end of that time the virulence of the mixtures was tested by inoculating 0·2 c.c. intradermally in a guinea-pig. All four mixtures gave reactions, the two made with larger quantity of virus being the greater; there was no difference between the mixtures containing convalescent serum and the normal human serum controls. Rivers, Berry and Rhoads (1930) were unable to obtain evidence of any neutralizing action on the part of convalescent serum when using mice as the test animal, but with rabbits there was some evidence of neutralization. The mixtures were inoculated intra-cerebrally in a dose of 0·25 c.c. after incubation for 5 hours at 37° C. and the criterion of infection or absence of it was the thermic response. Those rabbits receiving virus and normal serum showed a rise in temperature between 105° and 107° F., whereas the temperature of some of the rabbits receiving the virus and convalescent serum did not rise above 104° F. It is clear that whatever neutralizing power convalescent serum may possess it is of a low order and difficult to demonstrate, and the results do not suggest that convalescent serum would have any appreciable therapeutic value.

Immunizing Property of Virus Inactivated by Formalin.

A single experiment on this point (Bedson and Western, 1930) suggested that psittacosis virus inactivated by means of formalin was capable of inciting immunity in mice. A further experiment has been made. Spleens from infected mice were suspended in phosphate to give a 10% suspension. Its titre was 1/1000. Formalin was added to a concentration of 0·1% and stood in the refrigerator for 5 days, when it was used as the vaccine. The experiment can be conveniently resumed in a table (Table VI).

The titre of the suspension M. 162 was 1/20,000 (tested intradermally in the guinea-pig), so that mice 175 and 176 received at least 1000 infecting

TABLE VI.

Mouse.	Immunization.	Test of immunity.	Result.
173	*Nil*	Spleen suspension M. 162 0·5 c.c. 1/20 i. p.	Died 5th day.
174	„	„ „ 0·5 „ 1/100 „	„ 5th „
175	Three doses of 0·3 c.c. vaccine given intra-peritoneally at intervals of 6 days. Tested for immunity 10 days after last dose.	„ „ 0·5 „ 1/20 „	„ 4th „
176		„ „ 0·5 „ 1/20 „	Survived.
177		„ „ 0·5 „ 1/100 „	„
178		„ „ 0·5 „ 1/100 „	„
179		„ „ 0·5 „ 1/100 „	Died 6th day.

doses and the other three 200. Despite this stringent test 3 out of the 5 protected mice survived without having shown any obvious signs of illness, one of them after receiving the larger dose of virus, so that one is entitled to conclude that psittacosis virus rendered non-infecting by formalin is still capable of immunizing.

Cross-Immunity Tests with Various Strains of Virus.

In the above experiment one strain alone—"G.B."—had been used both in preparation of the vaccine and testing immunity. The 3 surviving mice were bled out and their pooled serums used for cross-immunity experiments. In the first experiment the pooled serum was put up against the homologous strain "G.B." and the parrot strain P. XV. Serum dilutions of 1/1 and 1/5 in phosphate were mixed with equal parts of the virus filtrates and stood at room temperature for 2 hours. Controls were put up with both viruses. Neutralization was tested for by inoculating 0·2 c.c. quantities of the mixtures intradermally in a guinea-pig. All the four mixtures containing the immune serum failed to give a skin reaction, whereas the two controls gave strong reactions.

In a second experiment the strain "J.S." was titrated in phosphate and in the presence of the immune mouse serum diluted 1 in 10. This was done by making two series of dilutions in phosphate, one of 1/2, 1/10, 1/100 and 1/1000, and a second of 1/1, 1/5, 1/50, 1/500. The virus used was a suspension of mouse spleen in broth. To each tube of the second series was then added an equal volume of immune serum diluted 1 in 5 and thoroughly mixed. After standing for two hours at room temperature, each of the mixtures was tested by inoculating 0·2 c.c. in the guinea-pig's skin. The result was as follows:

	Dilution of virus (spleen suspension).			
	1/2.	1/10.	1/100.	1/1000.
Virus in phosphate	+	±	∓	—
„ serum	—	—	—	—

It is clear, therefore, that the serum of mice immunized with one of our strains "G.B.," which came from budgerigars, was capable of neutralizing not only the homologous strain, but our two other strains P. XV and "J.S.," which came from a parrot and a Java sparrow respectively. Unfortunately no

similar experiment could be made with human strains as all ours had been lost, but two mice which had recovered from infection with one of our human strains resisted inoculation with strain "G.B." in a dose which killed the controls in five days—a finding in keeping with some of our earlier observations (Bedson and Western, 1930). It would appear, then, that the various strains of psittacosis virus are immunologically identical.

CONCLUSIONS.

1. The guinea-pig is susceptible to the virus of psittacosis, and this animal can be used for maintaining strains, or what is probably more important, for their titration.

2. Quantitative filter and centrifuge experiments indicate that psittacosis virus is relatively large.

3. The relation between the virus and the micro-organismal-like bodies present in virulent material is discussed.

4. Virulent mouse spleen loses its activity rapidly when stored in 50% glycerol, but much less rapidly when placed in M/50 phosphate pH 7·6. A phosphate suspension of mouse spleen stored at 6°C. maintained the virulence unaltered for 34 days. Beyond that time the virulence appears to fall off rapidly. Freezing would seem to be a satisfactory method of conserving the virus in normal spleen, at any rate for periods up to two months.

5. Evidence is produced in support of the immunological identity of strains of psittacosis virus of various origins.

We are grateful to the Yarrow Fund, London Hospital, and the Ministry of Health, for grants towards the expenses of this research.

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THE CONCENTRATION OF THE PROTECTIVE SUBSTANCE IN ANTIPOLIOMYELITIS SERUM.

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Received for publication November 17th, 1930.

A potent antiviral poliomyelitis serum has recently been produced in the horse by the intramuscular inoculation of large doses of living virus (Fairbrother, 1930 ; Fairbrother and Morgan, 1930). This immune serum was found to be as potent as several samples of convalescent human serum which were similarly tested. Although there is no doubt concerning the beneficial results obtained by the early administration of convalescent serum in man, the use of this therapeutic agent cannot be regarded as entirely satisfactory owing to the difficulty of obtaining supplies and to its variation in potency.

The success that has attended concentration of other therapeutic sera, both antitoxic and antibacterial, suggested that such a procedure, if applicable to the poliomyelitis antibody obtained from the horse, might surmount some of these difficulties attendant on the clinical application of the human poliomyelitis serum. The following work deals with the fractionation of various samples of immune horse-serum and the examination of the antiviral content of the different protein fractions by means of the neutralization test.

Before the production of a concentrated antiviral preparation could be undertaken it was necessary to determine which fraction of the serum proteins was associated with the poliomyelitic antibody. The division of the serum proteins into three groups is based largely on the early work of Fuld and Spiro (1900), and of Pick (1902). The former workers separated the serum globulin into two fractions by precipitation with ammonium sulphate. The protein precipitated between 28 and 33 per cent. volume of saturated ammonium sulphate solution they called euglobulin, while that precipitated between the limits 34 and 46 per cent. was called pseudoglobulin. Although such a separation into eu- and pseudoglobulin is by no means perfect owing to the overlapping of their precipitation limits, the division is convenient for practical purposes, and has been used almost exclusively for the routine preparation of the pseudoglobulins of diphtheria, tetanus and dysentery (Shiga) antitoxins.

Throughout this paper the protein thrown out of solution from a fivefold dilution of the immune serum by 33 per cent. volume of saturated ammonium sulphate solution has been termed for convenience "euglobulin." The same term has also been applied to the protein thrown out of solution by dialysis of the serum and to protein that is insoluble in a saturated solution of sodium chloride. That the whole of the protein precipitated under these different conditions is not classical euglobulin (Panum, 1851; Chick, 1914), is fully realized. It is, however, a practical nomenclature which is in extensive use

where different immune sera are concentrated for therapeutic and experimental purposes.

At the present time the generally accepted view, based largely on the early work of Dieudonné (1897), Seng (1899), Gibson (1906) and Ledingham (1907), is that antitoxins, when produced in the horse, are associated almost entirely with the water-soluble pseudoglobulin fraction of the serum. An almost negligible amount of antitoxin is found to be associated with the euglobulin fraction, and none is found in the serum albumin.

The immune bodies that are classified generally under the term "antibacterial" or "antiviral" possess somewhat different properties, and they are usually found associated with a sparingly soluble globulin which is thrown out of solution at a comparatively low concentration of ammonium sulphate.

The literature concerning the fractional precipitation of the antiviral and antibacterial immune bodies is scanty. The first successful concentration of an antiviral serum appears to have been made by Hartley (1914), who found that the specific immune bodies in the serum of Hill bulls of Northern India which had been immunized with the virus of Rinderpest were completely precipitated when subjected to dialysis or saturation with common salt. This work thus showed that the rinderpest immune body was similar to, or associated with, the serum euglobulin. Henseval (1919) has stated that in the serum of rabbits immunized with vaccinia virus, the antiviral substance is distributed throughout the serum proteins, the greatest antiviral activity being associated with the euglobulin fraction. Felton (1926, 1928) found that the pneumococcus antibody was mainly precipitated with a water-insoluble globulin, which was thrown out of solution by simple dilution with distilled water and adjustment to pH 5·0 or by dialysis. He has further shown (1930) that the protective value of pneumococcus immune serum runs parallel with the specific agglutinin, precipitin, complement-fixation and opsonic activities during concentration.

The conclusion may be drawn that while the antitoxins are mainly associated with the serum pseudoglobulin, the immune bodies which make up the antibacterial or antiviral components of an immune serum are largely precipitated with a water-insoluble serum globulin.

TECHNIQUE.

Neutralization tests.—The method adopted in the earlier work (1930) was followed. A small amount of the serum fraction was mixed with the virus emulsion, usually 0·4 c.c. of the supernatant fluid of a 0·5 per cent. cord emulsion, and saline was added to make a total volume of 1·0 c.c. The mixtures were then placed at 37° C. for 1½ to 2 hours, and left overnight at 4° C. After a final thorough mixing, 0·5 c.c. was inoculated intracerebally under ether anæsthesia into monkeys (*M. rhesus*).

The test virus, as in the previous work, was one of great potency, and the test dose represented at least 100 minimal infecting doses. Each experiment was controlled by the inoculation of a monkey with the same amount of virus mixed with saline instead of serum. The control animal invariably exhibited symptoms after an incubation period of 4 to 6 days, and the subsequent illness

terminated with typical paralysis and death (see Fairbrother, 1930). In the tables given below the controls are not included, as the results have been grouped separately under the different serum fractions. Consequently it is necessary to emphasize here the fact that in every instance a control animal was employed, and that with this strain of virus "Fl. M." the average incubation period for more than 50 control animals examined during this and other work was approximately 4 days. As additional controls, serum fractions obtained from other sources were examined in a similar manner; the results are included in Table I. In no case was protection obtained, although in certain instances a comparatively large amount of serum protein was added to the virus test dose. This fact supplies further evidence of the specific nature of the antiviral substances found in the serum of Horse No. 1 after immunization with the poliomyelitis virus.

TABLE I.

Serum.	Fraction.	Mgm. of protein per 1 c.c.	Mgm. of protein per dose.	Animal.	Result.
Reconcentrated diphtheria antitoxin	Pseudoglobulin	150‡	45	M 208	†9 days.
		150‡	45	M 326*	†8 „
Reconcentrated tetanus antitoxin	„	150‡	45	M 327*	†7 „
Commercial sample of Rosenow's concentrated anti-poliomyelitis serum	Euglobulin§	200	60	M 207	†8 „
		200	60	M 310	†7 „
Horse No. 1 (normal)	„	24	14·5	M 287	†5 „
„ „ 2 („)	Pseudoglobulin	20	6·0	M 333*	†6 „
„ „ 4 („)	Whole serum	80‡	32·0	M 424	†8 „

* See text; † meaning death; ‡ approximate values only; § see Fasting (1929).

In certain instances, owing to the shortage of monkeys, animals which had remained quite well after being used for previous neutralization tests were employed. It has been shown repeatedly that no immunity follows the intracerebral inoculation of serum-virus mixture into monkeys. While, in most of these cases, 40 to 50 days were allowed to elapse before an animal was used a second time, 30 days was the shortest period before any re-inoculation. In the tables given below all such animals are marked with an asterisk.

Protein estimations.—The protein estimations were carried out by means of the Zeiss immersion refractometer. The refractive index of the clear protein solution was determined, the solution was then boiled, the precipitated protein filtered off, and the refractive index of this filtrate (which contained sodium chloride) was found. The amount of protein in solution was then calculated from the difference between the values of the refractive index before and after heating, using the factor 0·220 per cent. of protein per scale division for solutions containing between 0 and 4 per cent. of protein; and the factor 0·217 per cent. for more than 4 per cent. of protein.

EXPERIMENTAL.

Euglobulin Fractions.

Preparation.—(A) The immune serum, without the addition of antiseptic, was dialysed for 4 days against running tap-water and against distilled water for a further 24 hours. A considerable precipitate of euglobulin was thrown out of the solution during the dialysis. The precipitate was removed by centrifuging the dialyzed serum, and was washed with distilled water to remove traces of the supernatant pseudoglobulin and albumen solution. The precipitate was then treated with normal saline equal in volume to half the original volume of serum. Although this protein suspension was made slightly alkaline (pH 8) by the addition of dilute sodium carbonate solution, a small part of the precipitate frequently remained undissolved. After standing at room temperature overnight the suspension was vigorously shaken for a few minutes, and the undissolved protein removed by filtration through a Seitz filter. In some of the preparations the serum after dialysis was adjusted to the iso-electric point of euglobulin (pH 4·5–5·0) in order to facilitate the more complete separation of this protein.

(B) Preparations of euglobulin were also made by salting out this protein with ammonium sulphate. To the serum diluted with water was added a saturated solution of ammonium sulphate, so that at the level 33 per cent. by volume of saturated ammonium sulphate solution the original serum had been diluted five times. The whole was well mixed, allowed to stand overnight, and the precipitate of euglobulin filtered off. After dialyzing to remove ammonium sulphate the euglobulin was dissolved in normal saline and filtered through a Seitz filter.

(C) The insolubility of euglobulin in saturated sodium chloride has been used to prepare a further specimen of euglobulin from the immune serum. To 100 c.c. of serum an equal volume of saturated ammonium sulphate solution was added; the whole was then thoroughly shaken and allowed to stand overnight. The combined precipitates of euglobulin and pseudoglobulin were filtered off and shaken with 100 c.c. of a saturated solution of sodium chloride. The undissolved euglobulin was filtered off, dialyzed free from salt, and finally dissolved in about one-third of the original volume of normal saline.

Pseudoglobulin Fractions.

Preparation.—(D) The euglobulin was removed from the immune serum as described above. When method (B) was used for the removal of euglobulin the lower levels of saturation with ammonium sulphate in different experiments were made 28,* 30, 34 and 36 per cent., since it is well known that some euglobulin escapes precipitation at 28 per cent., and may, moreover, not be completely precipitated even at 34 per cent. with ammonium sulphate. After removing the precipitates produced by these levels of salt concentration, the

* Throughout this paper the phrase "made x per cent. of saturation with ammonium sulphate" has been used to indicate the following: To $(100-x)$ parts of the serum by volume there were added x parts by volume of a saturated aqueous solution of ammonium sulphate.

solutions were all made 48 per cent. saturation with ammonium sulphate. The precipitate of pseudoglobulin produced under these conditions was filtered off, dialyzed free from ammonium sulphate, and made up to a definite volume with normal saline. In the preparations using method (A) for the removal of euglobulin, the dialyzed serum was always adjusted to pH 5·0 after dialysis to ensure the complete precipitation of euglobulin, and thus avoid any excessive contamination of the pseudoglobulin fraction.

In the earlier experiments pseudoglobulin, prepared as described above, frequently showed definite neutralizing power. The fact that the euglobulin of the immune serum possessed considerable antiviral properties suggested that the protective power of these pseudoglobulin preparations might be due to the presence of small amounts of euglobulin—a possibility to be expected in view of the overlapping of their precipitation limits. To eliminate any such contamination of the pseudoglobulin with euglobulin, a preparation of pseudoglobulin was reconcentrated by dissolving in its original volume of saturated sodium chloride solution, filtering and re-precipitating with an equal volume of saturated ammonium sulphate solution. A preparation of pseudoglobulin was also made by collecting the protein thrown out of solution between 40 and 46 per cent. volume of saturated ammonium sulphate solution. Under these conditions about 25 per cent. of the total serum proteins were precipitated in this fraction, which can be considered practically free from euglobulin.

Albumin Fractions.

Preparation.—(E) After removing the euglobulin by method (A) or (B) the pseudoglobulin was thrown out of solution by making the diluted serum 48 per cent. saturation with ammonium sulphate and filtering. The albumin was then precipitated by the addition of crystals of ammonium sulphate until the solution became saturated with this salt. The precipitate was filtered off, dialyzed free from ammonium sulphate, 0·8 per cent. of sodium chloride was added and the solution was then passed through a Seitz filter. In order to procure an albumin preparation free from traces of pseudoglobulin, the protein precipitated between the limits 54 and 60 per cent. volume of saturated ammonium sulphate solution was collected, dialyzed free from salt and dissolved in normal saline.

RESULTS.

Euglobulin Fractions (Table II).

A euglobulin fraction prepared by method A from the horse-serum taken before immunization (9. 5 . 29) showed no antiviral properties, the animal M 287 dying in exactly the same time as the control animal.

At the end of the first intramuscular immunization (see Fairbrother, 1930), an unfiltered euglobulin suspension prepared by the dialysis of the whole serum (method A) completely protected animals M 334 and M 311 against the standard dose of virus employed. Filtration of the suspension, after adding 0·85 per cent. sodium chloride, yielded a clear euglobulin solution, which was also strongly antiviral and contained only 1·1 per cent. of total protein.

TABLE II.—*Euglobulin Fractions.*

Date of bleeding.	Method of preparation.	Mgm. protein per c.c.	Mgm. protein per dose.	Monkey.	Result.
9.5.29 (before immunization)	A. Dialysis	24·0	14·5	M 287	†5 days.
9.8.29 (after 1st intramuscular course)	A. Dialysis, unfiltered, heated 56° ½ hour	...	...	M 311	0
		...	...	M 334	0
	Filtered	11·0	3·3	M 233	0
31.12.29 (after 2nd intramuscular course)	A. Dialysis	3·7	1·1	M 331*	0
	B. Salting-out with ammonium sulphate	14·0	0·35	M 443	†6 days.
		14·0	0·14	M 425	0
		19·2	5·7	M 320*	0
		13·7	4·1	M 319*	0
		3·0	0·90	M 318*	0
		2·2	0·32	M 324*	0
		16·0	4·0	M 354*	0
		16·0	1·6	M 355*	0
		16·0	0·8	M 356*	0
		16·0	0·4	M 371	0
		13·2	0·13	M 444	0
	C. Insoluble in saturated NaCl	2·2	0·82	M 420	0
		2·2	0·33	M 446	0
1.4.30 (after 3rd immunization; 1st intravenous course)	B. Salting-out with ammonium sulphate	5·2	1·5	M 290	0
		1·5	0·45	M 332*	†7 days.
		1·0	0·31	M 289	†7 „
	A. Dialysis	6·0	1·8	M 336*	†7 „

After the second intramuscular immunization (31.12.29) the serum yielded a euglobulin fraction (method A together with the additional precaution of adjusting the hydrogen ion concentration of the serum after dialysis to the isoelectric point of euglobulin) which protected animal M 331, although only 1·1 mgm. of the euglobulin protein was given with the virus. An attempt was made to find the minimum quantity of euglobulin prepared by this method that would neutralize the standard dose of virus (estimated to contain at least 100 minimal infective doses) used in these experiments. From Table II it will be seen that neutralization was complete with as little as 0·14 mgm. of this water-insoluble protein (animal M 425). No explanation can be offered for the incomplete neutralization of the virus when 0·35 mgm. of the protein was used in the test on animal M 443.

Euglobulin preparations were also made from the serum by salting out with ammonium sulphate. The levels of salt concentration at which the various preparations of euglobulin were thrown out of solution were 28, 34 and 36 per cent. volume of saturated ammonium sulphate solution. Under these conditions 12, 20 and 26 per cent. respectively of the total serum proteins were precipitated. The protein in each case was freed from the precipitating salt by dialysis, and redissolved in normal saline. The results of the neutralization tests (animals M 318, 319 and 320) showed that the euglobulin fractions collected between the limits 0 to 28, 0 to 34 and 0 to 36 per cent. respectively possessed antiviral properties. With the

intention of finding the minimum weight of this sparingly soluble protein which would neutralize the standard dose of virus, a preparation was made by collecting the protein fraction thrown out between 28 and 34 per cent. volume of saturated ammonium sulphate; the precipitate was freed from salt and redissolved in normal saline. In the first preparation the final strength of the protein solution was 0·217 per cent., and the serum dose, 0·15 c.c., contained only 0·32 mgm. of the protein. This quantity completely neutralized the 0·2 c.c. of 5 per cent. virus emulsion when the mixture was inoculated intracerebrally into animal M 324. Unfortunately, owing to the shortage of experimental animals at this time, this particular euglobulin preparation was not titrated to its limit of protection. A second preparation was made later from the same serum (bleeding 31 . 12 . 29), and a euglobulin solution containing 1·6 per cent. of protein was obtained. Decreasing quantities of this solution were then mixed with the 5 per cent. virus and tested for neutralizing power on animals M 354, 355, 356, 371 and 444. It will be seen from Table II that complete neutralization has taken place when 0·13 mgm. or more of the protein has been used.

A preparation of the serum euglobulin was also made from this bleeding (31 . 12 . 29) by the method described under C above. Decreasing doses of this euglobulin were then titrated against the standard dose of virus. The experiment as a whole was unsuccessful owing to some of the test animals dying of an intestinal infection, thus rendering the titration incomplete. Neutralization tests carried out on animals M 420 and 446 show that the globulin, insoluble in saturated salt solution, has considerable antiviral activity, for in the latter animal 0·33 mgm. of the protein completely neutralized the test dose of virus.

Euglobulin preparations were made from a later bleeding (1 . 4 . 30) when, after a rest of two months, the horse was further immunized with virus intravenously (Fairbrother and Morgan, 1930). By salting out with ammonium sulphate a preparation of euglobulin containing 0·52 per cent. protein was obtained; 1·56 mgm. of this euglobulin neutralized the virus (animal M 290), while the same volume of a five-fold dilution, *i. e.* 0·31 mgm., failed to protect the test animal M 289. Another preparation from the same serum failed to neutralize when 0·45 mgm. of the protein was used. By dialysis (method A) a euglobulin solution was obtained, and this also failed to neutralize when 1·8 mgm. of the protein was used (animal M 336).

As far as it is possible to judge from such a limited number of titrations, it would seem that after the resting period and the intravenous course of inoculations the euglobulin fraction possessed a lower protecting power than after the previous intramuscular courses; the serum euglobulin possessed a very marked antiviral power at the end of the second immunization.

Pseudoglobulin Fraction.

A pseudoglobulin fraction obtained from the horse-serum after the first immunization (9 . 8 . 29), by collecting the protein thrown out of solution between 30 and 48 per cent. saturation with ammonium sulphate solution, failed to neutralize the test dose of virus even in amounts as large as 60 mgm.

(animals M 204, 205 and 206, Table III). A later preparation of reconcentrated pseudoglobulin from the same serum neutralized the test dose of virus in the test animal M 308, but the same quantity of protein failed to protect another animal, M 309. It is difficult to explain the protection in animal M 312, the neutralization of the test dose of virus being apparently complete with only 10 mgm. of the same pseudoglobulin preparation. At the end of the second immunization (31.12.29) the pseudoglobulin showed a much greater neutralizing power. Although the protein was collected over varying ranges of salt concentration (28 to 46 per cent., 34 to 46 per cent., 36 to 46 per cent.), thus eliminating increasing amounts of euglobulin, the pseudoglobulin preparations appeared to possess quite definite neutralizing properties (animals 321, 322 and 323). The pseudoglobulin collected between the levels 40 and 46 per cent. saturated ammonium sulphate solution also showed neutralizing properties, as is seen from the rough titration of this protein solution on animals M 357, 358 and 369. Reconcentrated pseudoglobulin from the serum of a bleeding after the third immunization (1.4.30) also showed definite but weaker neutralizing properties (animals M 291 and 335).

TABLE III.—*Pseudoglobulin Fractions.*

Date of bleeding.	Method of preparation.	Mgm. protein per c.c.	Mgm. protein per dose.	Animal number.	Result.
9.8.29	Salting-out with $(NH_4)_2SO_4$ 30–48%	200	60	M 204	†10 days.
		200	30	M 205	† 8 „
		200	10	M 206	† 7 „
		200	50	M 308	0
		200	50	M 309	†15 days.
		200	10	M 312	0
31.12.29	28–48%	82·4	16·5	M 321	0
	34–48%	79·5	15·9	M 322*	0
	36–48%	70·5	14·0	M 323*	0
	40–46%	52·0	13·0	M 357*	0
	40–46%	52·0	5·2	M 358*	0
	40–46%	52·0	2·6	M 369	†12 days.
1.4.30	30–48% and reconcentration	200	60·0	M 291	0
		40	10·0	M 335	† 7 days.

In agreement with the results obtained with the euglobulin fraction, the pseudoglobulin prepared from the serum after the second immunization appears to be the most active of the three samples. Moreover, even when attempts have been made to eliminate euglobulin from the fractions a protective action was still demonstrated, indicating that a certain amount of the antiviral body is contained in the pseudoglobulin. This amount seems small compared with that found in the euglobulin, as judged by a comparison of the minimal quantities of pseudoglobulin and euglobulin necessary to protect against the test dose of virus.

Albumin Fraction.

The first preparations of albumin were made from the serum obtained on 1.4.30 (third immunization). A routine method was used and the protein

thrown out of solution between 48 per cent. and complete saturation was collected and tested for neutralizing properties in the animals M 292, 293 and 337. The animals receiving more than 30 mgm. of the protein were completely protected against the potent virus, while with 25 mgm. the disease ran an almost normal course and terminated fatally in 7 days. With a more potent immune serum (bleeding 31 . 12 . 29), 8 mgm. of the protein obtained between the same saturation limits completely neutralized the test dose of virus. Animals M 352, 353, 354 were given decreasing doses of the albumin separated between the saturation limits 54 and 60 per cent. It will be seen that neutralization has taken place with as little as 2·3 mgm. of protein, which, from its method of preparation, cannot contain any appreciable amount of eu- or pseudoglobulin.

As in the case of the pseudoglobulin, a comparison of the minimum amounts of protein conferring protection indicates that the antiviral content of the albumin is much smaller than that of the euglobulin fraction.

TABLE IV.—*Albumin Fractions.*

Date of bleeding.	Method of preparation.	Mgm. protein per c.c.	Mgm. protein per dose.	Animal number.	Result.
31 . 12 . 29	Ammonium sulphate 48%—saturation	27	8·1	M 325*	0
	54–60%	23	5·8	M 352*	0
	54–60%	23	2·3	M 353*	0
	54–60%	23	1·2	M 354	†4 days
1.4.30	48%—saturation	105	31·5	M 292	0
	48%— „	214	64·0	M 293	0
	48%— „	214	25·5	M 337*	†7 days

DISCUSSION.

The difficulty of making a hard and fast division of the serum globulin into euglobulin and pseudoglobulin fractions, together with the experimental difficulty of determining the exact concentration of antiviral substance in any protein fraction, suggests that these experimental results are calculated to give only a general idea of the proportional distribution of the antiviral substance throughout the serum proteins.

As far as it is possible to draw any conclusions of a general nature from these experimental results, the evidence indicates that the antiviral body is probably distributed throughout the serum, and is therefore contained in all the protein fractions. By gradually decreasing the amount of the different protein fractions added to the test dose of virus, it has been possible to obtain an approximate idea of the relative neutralizing power of the different serum proteins. It will be seen from the tables that approximately 0·13 mgm. of the serum euglobulin completely neutralizes the test dose of virus, while ten to fifteen times as much reconcentrated pseudoglobulin or albumin was required to produce the same degree of neutralization. Before immunization 14·5 mgm. of the serum euglobulin prepared by dialysis failed to neutralize the test dose of virus. All the protein fractions prepared by salting out up to

33 per cent. volume of saturated ammonium sulphate solution and termed for convenience in this paper "euglobulin" contain some pseudoglobulin. That this latter protein is not the essential source of the antiviral body is evident from the results obtained with the reconcentrated and carefully fractionated pseudoglobulin preparations. Furthermore, the protein thrown out of solution from the immune serum by simple dialysis or saturation with sodium chloride possesses strong antiviral properties as indicated by the neutralization test, and these fractions can, from their method of preparation, contain but traces of the water-soluble and salt-soluble classical pseudoglobulin. The presence of the greater part of the immune body in the more sparingly salt-soluble and water-insoluble globulin falls into line with the observations of other workers, who have found antibacterial and antiviral immune bodies in this globulin fraction. Although special precautions have been taken in this work to eliminate traces of euglobulin which might remain in certain of the pseudoglobulin preparations, a definite though slight neutralizing power has generally been obtained with these more soluble globulin fractions. In the same way albumin fractions, separated from the immune serum under conditions which preclude the possibility of all but insignificant traces of euglobulin and pseudoglobulin being present, are capable of neutralizing the virus.

There is some evidence that with certain immune sera the potency of the various protein fractions changes to some extent during the course of prolonged and intensive immunization. Thus, whereas early in the immunization a considerable proportion of the immune body may be in the protein precipitated by dialysis, by saturation with sodium chloride or by ammonium sulphate solution up to 34 per cent. saturation, later in the immunization it is to be found mainly associated with the soluble pseudoglobulin. Since all the protein fractions described in this paper have been obtained from the immune serum of one horse after a short immunization, the results must be considered in the light of the above evidence.

If further experience with the immune serum of other horses confirms our general conclusions, it would appear that for practical purposes a concentrated antiviral preparation, containing a maximum of the virus-neutralizing substance associated with the minimum of inert serum protein, can be obtained if the serum protein precipitated up to 34 per cent. volume of saturated ammonium sulphate is collected, and after dialysis, redissolved in normal saline. In consequence of the elimination of much non-specific protein, such a concentrated preparation should possess definite advantages, especially where treatment by the intraspinal route seriously limits the volume of the antibody solution that can be given.

SUMMARY.

From the experimental results obtained with the immune serum from one horse immunized with the living poliomyelitis virus, it would appear that the protein precipitated at a relatively low concentration of ammonium sulphate is uniformly much more potent in antiviral action per unit weight of protein than that precipitated at higher concentrations of this salt; and in conformity with this result the protein thrown out of solution by dialysis of the immune

serum, and the serum protein that is insoluble in saturated sodium chloride solution, have been shown to possess potent antiviral properties.

The evidence indicates that in this serum there was a general distribution of the antibody throughout the serum proteins, since carefully fractionated preparations of the pseudoglobulin and albumin possess definite although slight virus neutralizing properties.

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Since this paper was sent to Press a brief abstract of a paper read before the Association of American Pathologists at Chicago in March, 1929, and published in the *American Journal of Pathology*, **5**, 517, by E. R. Weyer, W. H. Park and E. J. Banzhaf, has come to our notice. These authors state that the poliomyelitis antibody is associated with the serum pseudoglobulin. The absence of protocols makes it impossible to compare their methods and results with our own.

ERRATUM.

IN the paper by F. Ridley (vol. xi, p. 225) the percentage of sugar in tears should read "0·065," and not "0·65" as given in the table.

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VOL. XI. No. 6] [DECEMBER, 1930.

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EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C.1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, January to December, post free, Two Pounds net.
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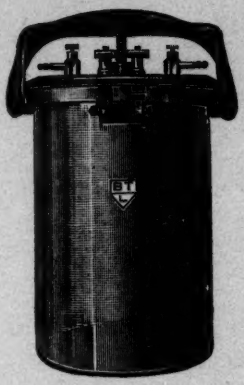

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